

ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 10



REDACTORES

Y. REENPÄÄ *A. KROGH* *E. LANGFELDT*
Helsinki *Kjöbenhavn* *Oslo*

G. LILJESTRAND (EDITOR)
Stockholm

COLLABORANTES

G. AHLGREN (Lund), Y. AIRILA (Helsinki), E. L. BACKMAN (Uppsala),
G. BLIX (Uppsala), J. BOCK (Kjöbenhavn), R. EGE (Kjöbenhavn),
H. v. EULER (Stockholm), U. S. v. EULER (Stockholm), A. FÖLLING
(Oslo), R. GRANIT (Stockholm), G. GÖTHLIN (Uppsala), E. HAMMARSTEN
(Stockholm), E. HANSEN (Kjöbenhavn), K. HANSEN (Oslo), E. HOHWÜ-
CHRISTENSEN (Stockholm), G. KAHLSON (Lund), F. LEEGAARD (Oslo),
J. LEHMANN (Göteborg), J. LINDHARD (Kjöbenhavn), E. LUNDSGAARD
(Kjöbenhavn), K. MÖLLER (Kjöbenhavn), R. NICCLAYSEN (Oslo), S. ÖR-
SKOV (Aarhus), A. V. SAHLSTEDT (Stockholm), F. SCHÖNHEYDER (Aarhus),
P. E. SIMOLA (Helsinki), T. TEORELL (Uppsala), H. THEORELL (Stockholm),
T. THUNBERG (Lund), A. WESTERLUND (Uppsala),
A. I. VIHTANEN (Helsinki)

STOCKHOLM 1945

*Reprinted with the permission of Acta Physiologica Scandinavica
Karolinska Instituturer, Stockholm*

JOHNSON REPRINT CORPORATION JOHNSON REPRINT COMPANY LIMITED
111 Fifth Avenue, New York, N.Y. 10003 Berkeley Square House, London, W.1

S. M. S. MEDICAL COLLEGE,

L. T. B. A. C. I.,

J. R. No... 5551..

Date... 15. NOV. 1965



First reprinting, 1964, Johnson Reprint Corporation

Printed in the United States of America

V O L. 10. I N D E X.

Fasc. 1. (2. VIII. 1945.)

	Pag.
Body Temperature and Capacity for Work. By ERLING ASMUSSEN and OVE BÖJE	1
On Serum Choline Esterase Activity in Experimental Liver Injury. By GUNNAR STEENSHOLT and HELGE VENNDT	23
The Influence of Adrenal Cortex on the Cholesterol Content in Rabbit Serum. By JÖRGEN HOFFMEYER	31
Contribution to Thunberg's Method of Estimating Citric Acid. By LARS HAGELSTAM	42
On the Activation of Pancreatic Lipase by Calcium Chloride at Varying pH. By FRITZ SCHÖNHEYDER and KIRSTEN VOLQVARTZ	62
Studies on Serum Phosphatase Activity in Relation to Experi- mental Biliary Obstruction in Rabbits. I. By OLAF JALLING, THOMAS LAURSEN and KIRSTEN VOLQVARTZ	70
Oxygen Supply and Tissue Growth in vitro. By ALBERT FISCHER, BØRGE FOGH and AAGE BOHUS JENSEN	78
The Osmotic Concentration of Human Lacrymal Fluid. By A. KROGH, C. G. LUND and K. PEDERSEN-BJERGAARD	88
Chromatographic Method for the Determination of Glutamic and Aspartic Acid in Mixtures of Different Amino Acids. By SVEN DARLING	91

Fasc. 2. (29. IX. 1945.)

The Presence of a Gastric Secretory Excitant in the Human Gastric and Duodenal Mucosa. By BÖRJE UVNÄS	97
An Experimental Study of Disorders in the Permeability of the Cerebral Vessels ("the Blood-Brain-Barrier") Produced by Chemical and Physicochemical Agents. By TORE BROMAN and A. LINDBERG-BROMAN	102
Gefässerweiternde Stoffe des Harns und Renin. Von ERNST WOLL- HEIM	126
A Micromethod for the Estimation of Phosphatidyl Ethanolamine in Nerve Tissue. By P. V. EDMAN and S. E. G. ÅQVIST	144
Kinetic and Thermodynamic Investigations on the Transamination Process. By SVEN DARLING	150

	Pag.
Peroxidation of Body Fat in Vitamin E Deficiency. By HENRIK DAM and HUMBERTO GRANADOS	162
Spectral Absorption of Bilirubin. By TORBEN K. WITH	172
The Diazo Reaction of Bilirubin. By TORBEN K. WITH	181
 Fasc. 3—4. (30. XI. 1945.)	
Obituary Notice. Erik Widmark 1889—1945. By G. LILJESTRAND	193
A Geiger-Müller Counter Arrangement for Biological Research. By J. AMBROSEN, B. MADSEN, J. OTTESEN and K. ZERAHN	195
The Measurement of Minute Samples with the Geiger-Müller Counter. By J. OTTESEN	214
The Effect of the Active Thyroid Substance on Metabolism and Other Functions in Guinea Pigs. By MARIE KROGH and ANNA-LOUISE LINDBERG	218
Metabolism in Different Parts of the Brain, Especially in the Epiphysis, Measured with Radioactive Phosphorus. By ULF BORELL and ÅKE ÖRSTRÖM	231
Electrical Impedance Properties of Surviving Gastric Mucosa of the Frog. By TORSTEN TEORELL and RICHARD WERSÄLL	243
Studies on the Lymphocyte Production. By ERIK ANDREASEN and JENS OTTESEN	258
Studies on the Blood Supply to Certain Regions in the Lumbar Part of the Spinal Cord. By ERIK KROGH	271
Studies on Nucleic Acid Metabolism in Plasma Cells. By J. BING, A. FAGRÆUS and B. THORELL	282
Studies on Dietary Gizzard Ulcers in Chicks. By HENRIK DAM and HARRY L. SEGAL	295
Studies on the Capacity of Serum to Bind Iron. By C. G. HOLMBERG and C.-B. LAURELL	307
On the Methylation of Guanidine Acetic Acid in the Animal Body. By GUNNAR STEENSHOLT	320
On the Action of some Adrenaline-like Substances on the Respiration of Suprarenal Tissue. By GUNNAR STEENSHOLT	327
On the Synthesis of Choline in the Animal Body. By GUNNAR STEENSHOLT	333
A Pulse Time Recorder. By SVEND JOHNSEN	342
Studies on the Functional Sphincter in the Cervix Uteri. By HANS FUCHS and AXEL WESTMAN	350
Bilirubin in Urine and Other Secretions apart from the Bile and in the Cerebrospinal and Eye Liquors. By TORBEN K. WITH	355
A Method for Direct Measurement of the Aortic Pressure in the Dog. By ARNE P. SKOUBY	366
The Morphology of the Pulse Curve of the Aorta. By ARNE P. SKOUBY	374
On the Presence in Extracts of Leaves and Plants of a Factor interfering with the Chemical Determination of Thiamin by the Method of Melnick and Field. By GUNNAR ÅGREN	381

Supplementum XXIX. On the Concentrations of Some Sulfanilamide Derivatives in different Organs and Tissue Structures. By STURE HELANDER.

Supplementum XXX. The Metabolic Effect and the Binding of Carbon Dioxide in Baker's Yeast. By KNUT M. BRANDT.

Supplementum XXXI. The Action Potential and Excitatory Effects of the small Ventral Root Fibres to Skeletal Muscle. By LARS LEKSELL.

Supplementum XXXII. A Dielectric Study of Polynucleotides. By GUNNAR JUNGNER.

Erratum.

p. 144 line 5 from below stands BRANTE 1940, read BLIX 1940. The corresponding reference p. 149 should be BLIX, G., Bioch. Z. 1940. 305. 129.

INDEX AUCTORUM.

	Pag.
AMBROSEN, J., B. MADSEN, J. OTTESEN, and K. ZERAHN, Counter Arrangement for Biological Research	195
ANDREASEN, E., and J. OTTESEN, Lymphocyte Production	258
ASMUSSEN, E., and O. BÖJE, Body Temperature and Capacity for Work	1
BING, J., A. FAGRÆUS, and B. THORELL, Nucleic Acid Metabolism in Plasma Cells	282
BOHUS JENSEN, A., A. FISCHER, and B. FOGH, Oxygen Supply and Tissue Growth in Vitro	78
BORELL, U., and Å. ÖRSTRÖM, Metabolism in Different Parts of the Brain	231
BROMAN, T., and A. M. LINDBERG-BROMAN, Permeability of Cerebral Vessels	102
BÖJE, O., and E. ASMUSSEN, Body Temperature and Capacity for Work	1
DAM, H., and H. GRANADOS, Peroxidation of Body Fat	162
DAM, H., and H. L. SEGAL, Dietary Gizzard Ulcers in Chicks	295
DARLING, S., Determination of Glutamic and Aspartic Acid	91
DARLING, S., The Transamination Process	150
EDMAN, P. V., and S. E. G. Åqvist, Phosphatidyl Ethanolamine in Nerve Tissue	144
FAGRÆUS, A., J. BING, and B. THORELL, Nucleic Acid Metabolism in Plasma Cells	282
FISCHER, A., B. FOGH, and A. BOHUS JENSEN, Oxygen Supply and Tissue Growth in Vitro	78
FOGH, B., A. FISCHER, and A. BOHUS JENSEN, Oxygen Supply and Tissue Growth in Vitro	78
FUCHS, H., and A. WESTMAN, The Functional Sphincter in the Cervix Uteri	350
GRANADOS, H., and H. DAM, Peroxidation of Body Fat	162
HAGELSTAM, L., Thunberg's Method of Estimating Citric Acid Content	42
HOFFMEYER, J., Influence of Adrenal Cortex on the Cholesterol Content	31
HOLMBERG, C. G., and C.-B. LAURELL, Capacity of Serum to bind Iron	307
JALLING, O., T. LAURSEN, and K. VOLQVARTZ, Serum Phosphatase Activity	70

	Pag.
JOHNSEN, S., A Pulse Time Recorder	342
KROGH, A., C. G. LUND, and K. PEDERSEN-BJERGAARD, Osmotic Concentration of Human Lacrymal Fluid	88
KROGH, E., Blood Supply in Spinal Cord	271
KROGH, M., and A.-L. LINDBERG, Thyroid Substance on Metabolism	218
LAURELL, C.-B., and C. G. HOLMBERG, Capacity of Serum to bind Iron	307
LAURSEN, T., O. JALLING, and K. VOLQVARTZ, Serum Phosphatase Activity	70
LILJESTRAND, G., Obituary Notice. Erik Widmark	193
LINDBERG, A. L., and M. KROGH, Thyroid Substance on Metabolism	218
LINDBERG-BROMAN, A.-M., and T. BROMAN, Permeability of Cerebral Vessels	102
LUND, C. G., A. KROGH, and K. PEDERSEN-BJERGAARD, Osmotic Concentration of Human Lacrymal Fluid	88
MADSEN, B., J. AMBROSEN, J. OTTESEN, and K. ZERAHN, Counter Arrangement for Biological Research	195
OTTESEN, J., J. AMBROSEN, B. MADSEN, and K. ZERAHN, Counter Arrangement for Biological Research	195
OTTESEN, J., Samples with the Geiger-Müller Counter	214
OTTESEN, J., and E. ANDREASEN, Lymphocyte Production	258
PEDERSEN-BJERGAARD, K., A. KROGH, and C. G. LUND, Osmotic Concentration of Human Lacrymal Fluid	88
SCHÖNHEYDER, F., and K. VOLQVARTZ, Activation of Pancreatic Lipase by Calcium Chloride	62
SEGAL, H. L., and H. DAM, Dietary Gizzard Ulcers in Chicks	295
SKOUBY, A. P., Measurement of Aortic Pressure	366
SKOUBY, A. P., Morphology of Pulse Curve	374
STEEENSHOLT, G., and H. VENNDT, Serum Choline Esterase Activity	23
STEEENSHOLT, G., Methylation of Guanidine Acetic Acid	320
STEEENSHOLT, G., Action of Adrenaline-like Substances	327
STEEENSHOLT, G., Synthesis of Choline	333
TEORELL, T., and R. WERSÄLL, Properties of surviving Gastric Mucosa	243
THORELL, B., J. BING and A. FAGRÆUS, Nucleic Acid Metabolism in Plasma Cells	282
UVNÄS, B., Gastric Secretory Excitant	97
VENNDT, H., and G. STEENSHOLT, Serum Choline Esterase Activity	23
WERSÄLL, R., and T. TEORELL, Properties of surviving Gastric Mucosa	243
WESTMAN, A., and H. FUCHS, Functional Sphincter in the Cervix Uteri	350
WITH, T. K., Spectral Absorption of Bilirubin	172
WITH, T. K., Diazo Reaction of Bilirubin	181
WITH, T. K., Bilirubin in Urine and other Secretions	355
WOLLHEIM, E., Gefässerweiternde Stoffe des Harns und Renin ..	126

	Pag.
VOLQVARTZ, K., and F. SCHÖNHEYDER, Activation of Pancreatic Lipase by Calcium Chloride	62
VOLQVARTZ, K., O. JALLING, and T. LAURSEN, Serum Phosphatase Activity	70
ZERAHN, K., J. AMBROSEN, B. MADSEN, and J. OTTESEN, Counter Arrangement for Biological Research	195
ÅGREN, G., Chemical Determination of Thiamin	381
Åqvist, S. E. G., and P. V. EDMAN, Phosphatidyl Ethanolamine in Nerve Tissue	144
ÖRSTRÖM, Å., and U. BORELL, Metabolism in Different Parts of the Brain	231

From the Laboratory for the Theory of Gymnastics,
University of Copenhagen.

Body Temperature and Capacity for Work.

By

ERLING ASMUSSEN and OVE BØJE.

Received 23 March 1945.

Muscular exercise will produce a rise in body temperature as demonstrated by numerous investigators (JÜRGENSEN (1873), PEMBREY and NICOL (1898), ZUNTS and coll. (1901 and 1906), BENEDICT and SNELL (1902), HILL and FLACK (1907), LINDHARD (1910), CHRISTENSEN (1931), NIELSEN (1938) a. o.). The cause of this is the increased production of heat in the working muscles. With few exceptions the increase in temperature has been looked upon as a sign of failing temperature regulation, endangering the continuation of work. ZUNTZ and coll., however, called attention to the fact that the higher temperature during work might decrease the reaction time of nerves and muscles and thus increase the ability to work, and they mention that the subjective feeling of stiffness and numbness in the muscles at the onset of work disappears after a while as the body temperature rises. From another point of view BARCROFT and KING (1909) observe that an increased temperature of blood and muscles makes the dissociation of oxyhaemoglobin faster and more complete, thus facilitating the oxidative processes in the muscles during work.

These effects of a rise in body temperature were believed to be incidental and not essential for the efficient performance of work, because the rise in temperature was assumed to be the sign of an impaired heat regulation. The work of NIELSEN (1938), however, threw new light on the subject by demonstrating, that — inside a wide range — the rectal temperature during exercise rises to a constant level, dependent on the rate of work, but in-

dependent of the environmental temperature, humidity and air movement. Such an accurately regulated temperature during work can hardly be believed to be incidental, and NIELSEN therefore makes the assumption that the higher body temperature during exercise in some way or other benefits the performance of work. He also suggests a way in which to demonstrate this, i. e. by measuring the maximum capacity for work of subjects at different body temperatures.

Besides the scientific problems it raises, the question whether the higher temperature during work is an advantage or not, has a practical aspect too, viz. in sports, especially in athletics, where for quite a long time it has been well known that the best results could be achieved only after a preliminary period of "warming-up". But even where the term "warming-up" was used, the beneficial effect was generally not ascribed to any actual higher temperature, but rather vaguely to a "softening" of joints and muscles setting in on being moved, or to the acceleration of circulation and respiration occurring during the preliminary exercises.

It is the purpose of this paper to find out whether a higher temperature as such has a measurable beneficial effect on physical performances, as suggested by NIELSEN (1938). We shall therefore study also the effect of *passive* warming of the organism, on the work capacity. Finally we shall try to elucidate by which means a higher temperature benefits the work.

Methods.

The *work* consisted in riding a stationary bicycle, braked so as to provide a suitable resistance. For our purpose it was convenient that the resistance per revolution was constant at all speeds so that the subject might at any time choose the optimum speed at which to work. This is not feasible with the KROGH bicycle ergometer usually employed in this laboratory, as in this machine the resistance to be overcome per fly-wheel revolution rapidly diminishes with speeds exceeding about 175 rev/min., and the automatic regulation, introduced by KROGH acts too slowly to compensate for this during the short time of work. Instead of the electric brake of the KROGH ergometer we therefore employed a simple friction brake, which could easily be fitted on to the bicycle ergometer available. (See fig. 1.) The leather strap (l) could be given such a breadth, that the spring balance (s) would always be in the zero position, when the wheel was moving. The friction was found to be constant even at speeds of more than 600 rev/min.

As a measure of work capacity the time within which a certain amount of work could be done was used. We used two standard performances, one consisting of 35 turns of the pedals, giving a total amount of work performed of 956 mkg, and another consisting of 450 turns, giving a total output of 9,860 mkg. The former lasted 12 to 15 seconds, was performed mainly anaerobically and simulates a 100 m sprint. The second lasted 4 to 5 minutes, making serious demands on the respiratory and circulatory systems, and simulates a 1,500 m run.

In some cases the maximum power of the calf muscles was measured by means of two dynamometers (COLLIN), fixed to the floor at each side of the foot of the subject and connected by a chain across the knee of the sitting subject. The "work" then consisted in a maximal plantar

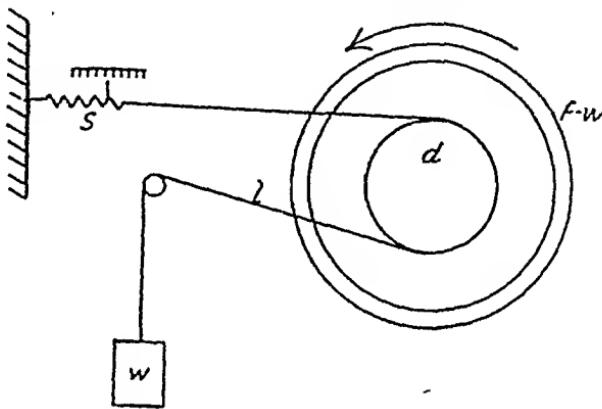


Fig. 1. Arrangement for frictional braking of bicycle ergometer.

- f-w fly-wheel of the Krogh bicycle ergometer.
- d dash-pot of brass, fixed on the fly-wheel.
- l leather strap.
- w weight.
- s spring balance.

Work per revolution of wheel: periphery of $d \times (W - \text{reading on } s)$.

flexion of the ankle joint. In still other cases the maximum work performed by a flexion of the elbow joint was determined by a "HILL'S fly-wheel", as modified in this laboratory (HANSEN and LINDHARD (1923)).

The preliminary work was performed on the bicycle ergometer at an established rate for a fixed time.

The rectal temperature was measured by an ordinary maximum thermometer; in some cases, where a continuous registration was deemed necessary, by a thermocouple in the rectum. The constant temperature for the corresponding thermocouple was furnished by water at about 32° in a DEWAR's flask.

Muscle temperatures of the working muscles were determined by means of a thermoneedle, a hypodermic steel cannula being one metal, and a concentric, insulated constantan wire the other. They were soldered together at the bevel of the cannula. (See BUCHTHAL, HÖNCKE

and LINDHARD (1945)). Also in this case the "constant" thermocouple was kept at about 32° in a waterfilled DEWAR's flask.

Determinations of metabolism were performed by the DOUGLAS bag method. Estimations of the blood lactate from finger blood were made according to EDWARDS (1938).

The subjects were healthy men in the age of 21 to 39 years. Some of them were active sportsmen — in a few supplementary investigations professional bicyclists of world reputation were used — others had not gone in for sport of any kind, or they were at the beginning of the experimental period utterly untrained. As the results turned out to be independent of their former records, no attempt at a classification was made.

It may be worth mentioning that none of the subjects knew what was the actual purpose of the experiments — at least not in the beginning. They were told that we were going to determine the effect of fatigue caused by the preliminary work on the work capacity. We are convinced that in this way possible psychic effects which might have accounted for the better performance when "warmed up", were eliminated.

Results.

The results from a series of experiments on four subjects are averaged (each figure represents the mean of 6 to 8 single determinations) and presented in table 1. The subjects were: H. A. 31 years, 176 cm, 71 kg; J. E. 25 years, 178 cm, 76 kg; K. G. 26 years, 175 cm, 76 kg and F. J. 29 years, 184 cm, 85 kg.

The columns marked "cold" are to be compared with the columns marked "warm". Preceding the work in the "cold" condition was a rest period of 30 min. duration. In the "warm" experiments the rest period was followed by a preliminary work for 30 min. at the rate of 660 mkg/min. Table 1 shows, that the

Table 1.
30 Min. of Preliminary Work.

Subj.	35 revolutions (956 mkg)					450 revolutions (9,860 mkg)				
	"Cold"		"Warm"		$\frac{I-II}{I} \times 100$	"Cold"		"Warm"		$\frac{I-II}{I} \times 100$
	Rectal temp.	I Time	Rectal temp.	II Time		Rectal temp.	I Time	Rectal temp.	II Time	
H. A. .	37.3°	14.4"	38.0°	13.9"	3.5	37.3°	5'39.1"	38.05°	5'29.8"	2.7
J. E. .	37.1°	14.1"	37.8°	13.3"	5.7	37.1°	4'39.7"	37.8°	4'26.2"	4.8
K. G. .	37.0°	14.4"	37.85°	13.5"	6.3	36.9°	5'19.0"	37.9°	5'09.4"	3.0
F. J. .	36.9°	13.7"	37.8°	12.6"	8.0	37.0°	4'48.5"	37.8°	4'32.5"	5.5

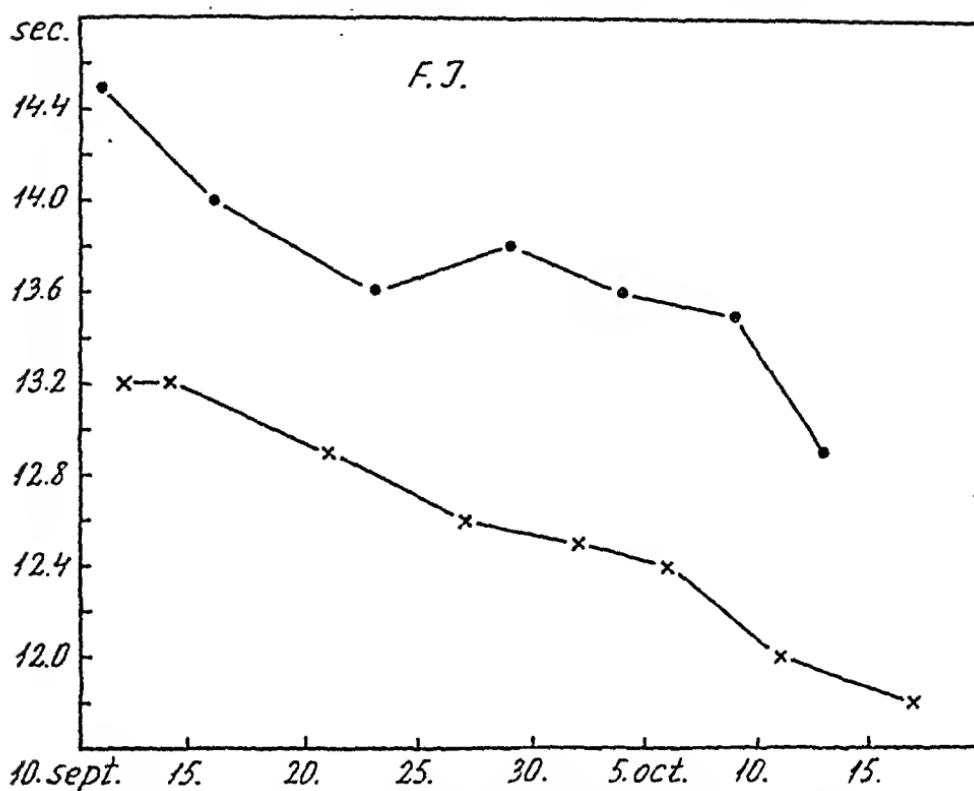


Fig. 2. Performance time for sprint (956 mkg), plotted against date of experiment. (Subj. F. J.)

●—● Without preliminary work.

×—× After preliminary work. (660 mkg/min. for 30 min.)

preliminary work increased the rectal temperature by about 0.8° . The time needed for performing the work was shorter when the subjects were "warm" than when they were "cold". The differences are calculated as per cent of the "cold" values $\left(\frac{I-II}{I} \times 100 \right)$ and presented in the table. It will be seen that the warming-up by the preliminary work has improved the results by between 2.7 and 8.0 per cent.

In fig. 2 the individual values for F. J. in performing the brief performance are plotted against the successive experimental days. All the results from the "cold" experiments are connected by lines as are all the results from the "warm" experiments. There is a general trend towards better results during the experimental period, which is due to the effect of training. But besides this fig. 2 shows that the results from the "warm" experiments are always better than the corresponding "cold" results.

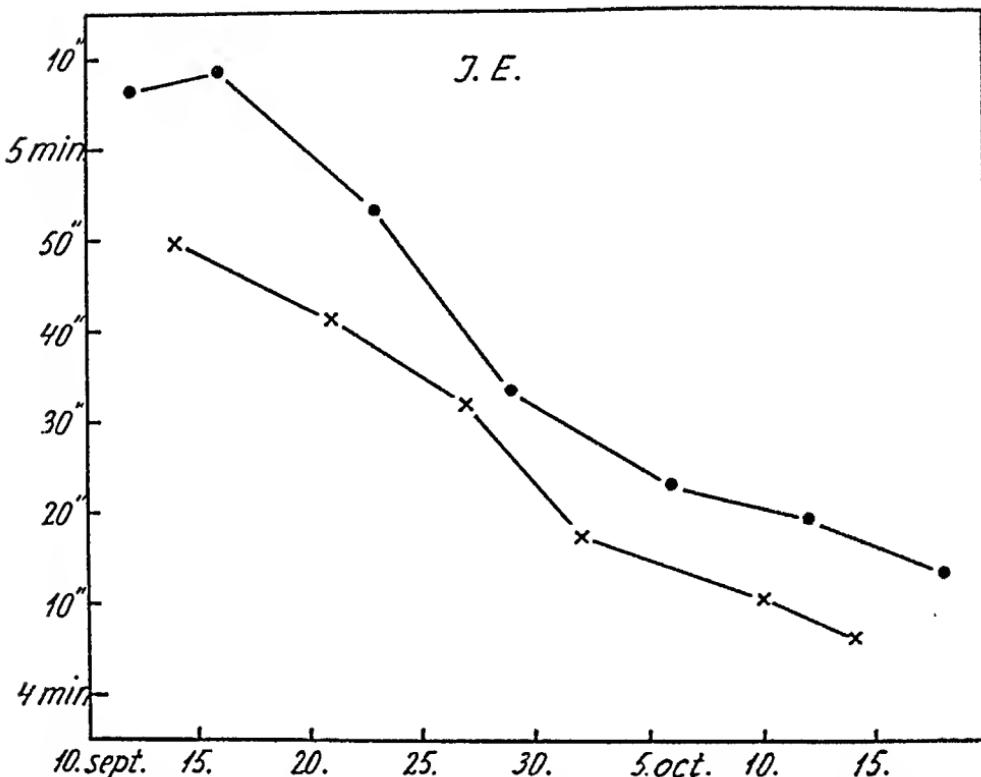


Fig. 3. Performance time for longer work (9,860 mkg), plotted against date of experiment. (Subj. J. E.)

● — ● Without preliminary work.

× — × After preliminary work. (660 mkg/min. for 30 min.)

The same is the case with the long-time work, as fig. 3 illustrates (subj. J. E.). Also in this kind of work there is a considerable effect of training throughout the experimental period, but the "warm" experiments always give better results than the "cold" ones.

In the case of the longer work the beneficial effect of the warming-up might be believed to be limited to the very first part of the work only, and that later on the capacity might be uninfluenced by the preliminary work. That this is not so is evident from table 2, in which the time for 100 pedal turns is calculated and averaged for the same four subjects as in table 1 for every hundred turns up to 400, and for the last 50, from 401 to 450.

It will be noticed that with the exception of the first hundred, the intermediary times are best when the subjects are "warm". The time for the first hundred revolutions is more apt to depend

on the temperamental state of the subjects than the later ones, as the subjects were allowed to plan their work according to their own minds. J. E., for instance, rode at an almost constant speed throughout the work, whereas the others apparently preferred to ride faster at the start, but consequently grew tired and had to slow down later on.

Table 2.
Time per 100 Revolutions.

Subj.	"Cold"					"Warm"				
	1— 100	101— 200	201— 300	301— 400	401— 450	1— 100	101— 200	201— 300	301— 400	301— 350
H. A.	1.04'	1.29'	1.38'	1.34'	1.14'	1.06'	1.28'	1.29'	1.28'	1.13'
J. E.	1.00'	1.04'	1.06'	1.06'	1.02'	0.94'	0.97'	1.01'	1.03'	0.97'
K. G.	1.07'	1.20'	1.23'	1.26'	1.06'	1.12'	1.13'	1.17'	1.20'	1.08'
F. J.	0.93'	1.11'	1.12'	1.16'	1.06'	0.98'	0.98'	1.06'	1.05'	0.95'

The beneficial effect of a preliminary work on the work capacity might be due to the increased temperature in the body, or it might be due to other physiological changes accompanying muscular exercise, e. g. of circulatory, respiratory or hormonal character. In order to investigate this problem experiments were performed in which the body temperature was raised *passively*. In this way it should be possible to exclude such additional factors from consideration and to investigate the effect of temperature alone on the capacity for work.

The heating of the organism was performed by means of short wave radio diathermy¹ or — in a few experiments — by means of a hot shower (47° C.) for about 10 minutes. In the experiments with radio diathermy large heating pads were placed under the buttocks and over the thighs, or the subject was placed on a special bed with two built-in heating pads, furnished by the makers of the diathermy machine.

The results from these experiments on the same four subjects as used earlier are averaged in table 3. (4 to 6 single determinations of each kind on each subject.)

It is evident, that also a passive heating of the body is able to improve the performances, both of the short-time and of the long-time type.

¹ We are much indebted to Mr. M. P. PETERSEN, civil engineer, who kindly placed a diathermy machine at our disposal.

Table 3.
Radio Diathermy.

Subj.	35 revolutions (956 mkg)					450 revolutions (9,860 mkg)				
	"Cold"		"Warm"		$\frac{I-II}{I} \times 100$	"Cold"		"Warm"		$\frac{I-II}{I} \times 100$
	Rectal temp.	I Time	Rectal temp.	II Time		Rectal temp.	I Time	Rectal temp.	II Time	
H. A. .	37.3°	14.3'	38.8°	13.6''	4.9	—	—	—	—	—
J. E. .	37.3°	13.6''	38.7°	12.8''	5.9	37.1°	4'26.9''	38.6°	4'11.4''	5.8
K. G. .	36.8°	13.7''	38.1°	12.8''	6.6	37.2°	4'49.7''	38.5°	4'38.3''	3.9
F. J. .	36.9°	12.9''	38.6°	12.3''	4.7	36.8°	4'53.8''	38.6°	4'31.4''	7.6

As radio diathermy heats the body from inside it greatly resembles the natural warming-up by means of muscular movements. Hot water, as used in our experiments with hot showers, heats the body from the outside. It was quite possible, that the differences in skin temperature, blood distribution etc. after the two modes of warming-up might prove to act differently on the work capacity. The results from experiments on two subjects, O. B. (39 years, 183 cm 78 kg) (5 experiments) and J. E. (data above, 4 experiments) show (table 4), that a 10 minutes' hot shower bath is able to raise the rectal temperature by 0.5° to 0.6° and that the performances as usual were better in the warm condition.

Table 4.
Hot Bath.

Subj.	35 revolutions (956 mkg)				
	"Cold"		"Warm"		$\frac{I-II}{I} \times 100$
	Rectal temp.	I Time	Rectal temp.	II Time	
O. B.	37.3°	15.4''	37.9°	14.3''	7.2
J. E.	37.1°	13.9''	37.6°	13.2''	5.0

As a third way of heating the subjects massage might be mentioned. Actual measurements of muscle temperatures after massage (BUCHTHAL, HÖNCKE and LINDHARD, personal communication) show, however, that very little can be reached in this way. Our results with two subjects (P. K. 23 years, 181 cm,

74 kg and P. L. 21 years, 181 cm 74 kg), who previous to work were vigorously massaged on legs and buttocks for 15 minutes by a strong masseur, are in good agreement with this observation. The results (from 7 experiments of each kind on each subject), averaged in table 5, show that massage had no or only very little effect on the two kinds of work investigated.

Table 5.

Subj.	35 revolutions (956 mkg)		450 revolutions (9,860 mkg)	
	After rest	After 15 min. massage	After rest	After 15 min. massage
P. K.	15.1"	14.9"	4'47.9"	4'47.2"
P. L.	13.7"	13.7"	5' 2.5"	4'52.3"

For the explanation of the results of experiments mentioned on the preceding pages it is essential to know not only the rectal temperature, but also the temperature of the working muscles. Muscle temperatures may deviate considerably from the rectal temperature, and they rise more rapidly during work than does the rectal temperature as shown recently by BUCHTHAL, HÖNCKE and LINDHARD (1945). This is demonstrated by fig. 4, in which the rectal temperature and the temperature of *M. vastus lateralis* are plotted against time from the onset of work. The work — on the bicycle ergometer — was at the rate of 660 mkg/min. The rectal temperature could be followed continually by means of a thermo-couple inserted about 20 cm into the rectum. For each measurement of the muscle temperature the subject had to stop for a moment while the thermo-needle was thrust into the lateral vastus muscle. This experiment, which was confirmed by experiments also on other subjects, shows that the muscle temperature in the active muscles rises almost abruptly and that after about 10 minutes a level is reached, which is maintained during the remainder of the working period (in one subject it was measured over a period of 70 minutes). The rise in the rectal temperature is lesser and slower, a level generally being reached after about 30 minutes, as shown by NIELSEN (1938).

The fact that the muscle temperature rises considerably faster during work than does the rectal temperature allows a differentia-

tion between the effect of the higher rectal temperature and the effect of the raised local temperature in the active muscles: After 5 minutes of preliminary work (660 mkg/min.) the muscle temperature is increased to more than 38° , whereas during the same time the rectal temperature only increases by 0.1 to 0.2° C. After 30 minutes of preliminary work the rectal temperature, too, has

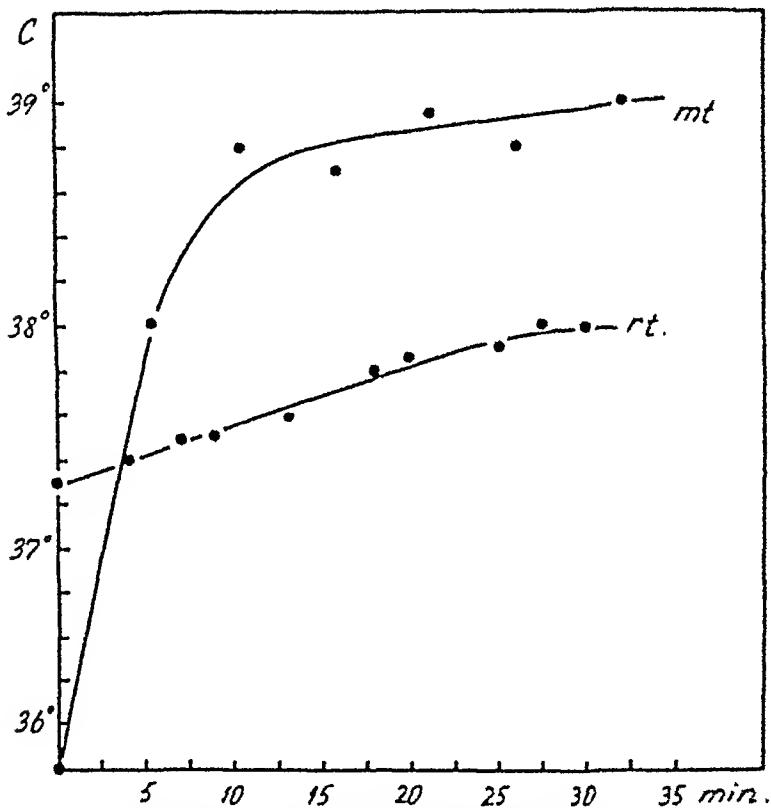


Fig. 4. Temperature measured in lateral vastus muscle (mt) and in rectum (rt) during a work of 660 mkg/min. (Subj. J. E.)

undergone a considerable increase, while the further increase in muscle temperature is relatively small. Roughly spoken it may be said, that 5 minutes of preliminary work raises the muscle temperature only, while 30 minutes of work raises both rectal and muscle temperature.

The effect of a preliminary work of 5, respectively 30 minutes duration on our short-time standard performance (35 pedal turns) is demonstrated in table 6. The figures are averages from several (5 to 9) experiments.

Table 6.

Subj.	After 30 min. rest			After 5 min. "warming-up"			After 30 min. "warming-up"		
	Rectal temp.	Muscle temp.	Time	Rectal temp.	Muscle temp.	Time	Rectal temp.	Muscle temp.	Time
H. A. ...	37.2°	37.0°	14.0''	37.3°	38.5°	13.4''	37.8°	39.4°	13.2''
O. B. ...	37.1	36.2	15.6''	37.3	38.2	14.6''	38.2°	39.2°	13.8''
J. E. ...	37.0°	37.1°	13.8''	37.1°	38.3°	13.1''	37.8°	38.9°	12.9''
F. J. ...	37.1°	37.5°	12.6''	37.2°	38.5°	12.3''	37.7°	39.0°	11.9''

Table 6 shows that 5 minutes of preliminary work greatly improves the performance, although the rectal temperature is only slightly increased. A further increase in temperature, both muscular and rectal, as will be found when the preliminary work has lasted 30 min. improves the results further still, though relatively slightly compared with the improvement after the first 5 minutes of work. Similar results were obtained also for the long-time performance of 450 revolutions.

A graphic representation of a series of corresponding experiments on K. G. is presented in fig. 5. The preliminary work, here at the rate of 985 mkg/min., lasted in the various experiments from 5 to 50 minutes. Immediately after this the rectal and the muscle temperature were measured, and the standard performance of 35 revolutions (956 mkg) was begun. The time needed for this, and the temperatures are plotted against the duration of the preliminary work in minutes. Fig. 5 shows that the main effect of the warming-up is achieved when the muscle temperature has reached its level, i. e. after 10 to 15 minutes, and that the further, slow increase in rectal temperature has but little effect on the performance time.

According to NIELSEN (1938), the rectal temperature is closely related to the intensity of work. So is presumably the temperature of the working muscles. It was of interest to see whether the very high temperatures that can be reached only after strenuous work, were still able to improve the capacity for work, or whether fatigue resulting from the preliminary work might obscure or outweigh the effect. A series of experiments therefore was performed on J. E. in which the intensity of the preliminary work varied between 200 mkg/min. and 1,640 mkg/min. In all experiments the duration of the preliminary work was 30 min. The heaviest work

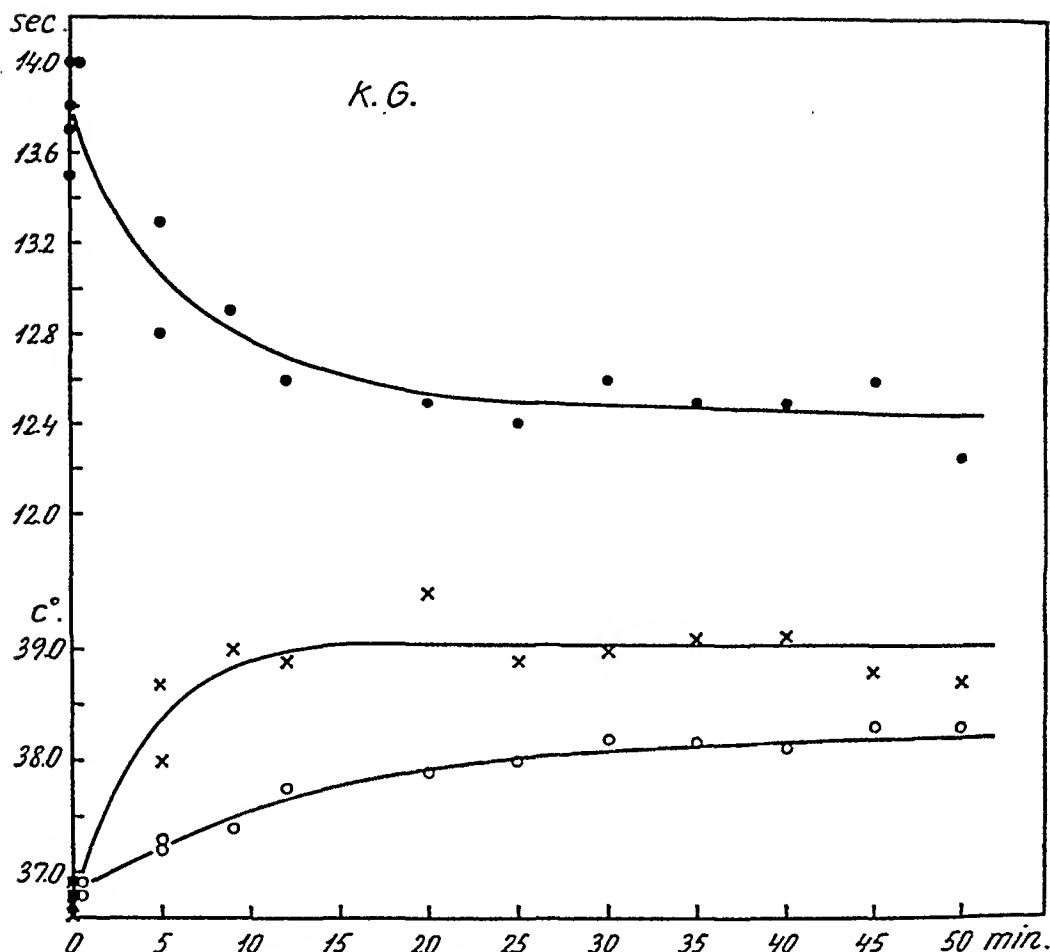


Fig. 5.

● — ● Performance time for sprint (956 mkg).

× — × Temperature of lateral vastus muscle.

○ — ○ Rectal temperature.

Abscissa: Duration of preliminary work (985 mkg/min.) (Subj. K. G.)

— 1,640 mkg/min. — was the maximum work this strong and well trained subject could perform. The results are plotted in fig. 6. It will be seen that both rectal and muscle temperatures increase with increasing intensity of the preliminary work, and further, that the time needed to perform the standard work of 35 revolutions (956 mkg) decreases steadily.

The effect of fatigue resulting from the preliminary work seems to be negligible in this kind of work, and a close relationship between temperature and work capacity is found.

The correlation between temperature and work capacity, as expressed by the time required to perform 956 mkg, appears in-

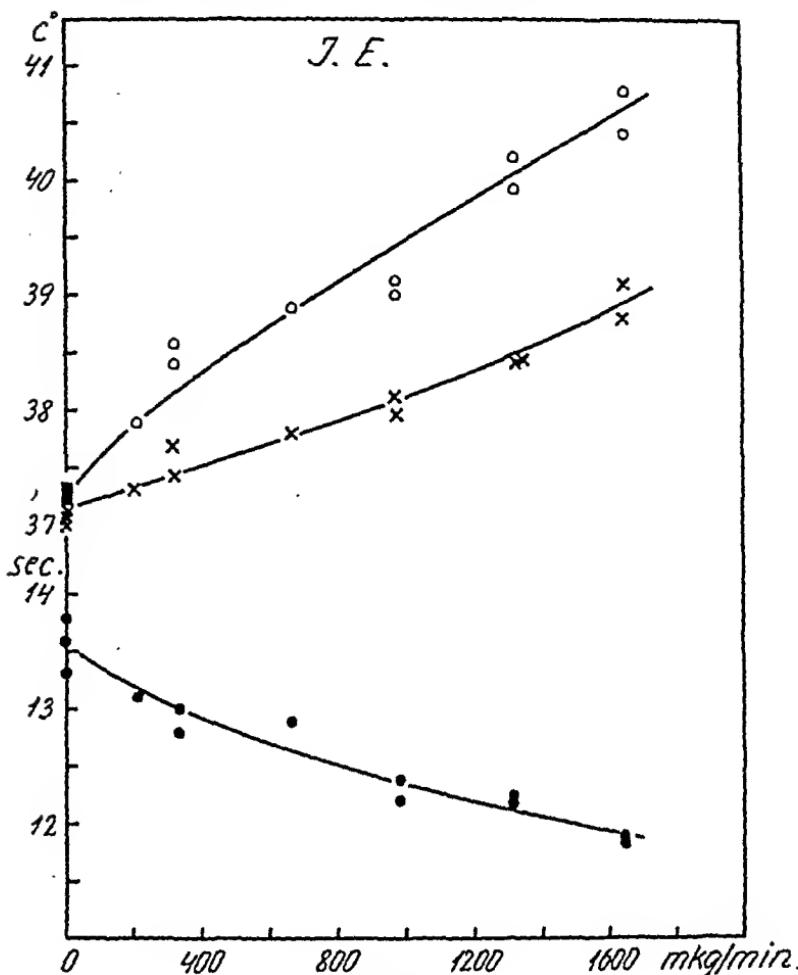


Fig. 6.

● — ● Performance time for sprint (956 mkg).

× — × Rectal temperature.

○ — ○ Temperature of lateral vastus muscle.

Abscissa: Intensity of preliminary work (30 min.) (Subj. J. E.)

complete, when the time of performance is plotted against the rectal temperature, because, as explained above, the performance time decreases considerably already after 5 to 10 minutes of preliminary work, during which time the rectal temperature only increases a few tenths of a centigrade. Between the muscle temperatures and the performance time, however, there seems to be a complete correlation inside a rather large range.

This is demonstrated by fig. 7, in which results from the short standard work on J.E. are plotted against muscle temperature (lateral vastus). Fig. 7 shows that in the range from 36.5° to 40.8° , the time used to perform the work decreases steadily, in-

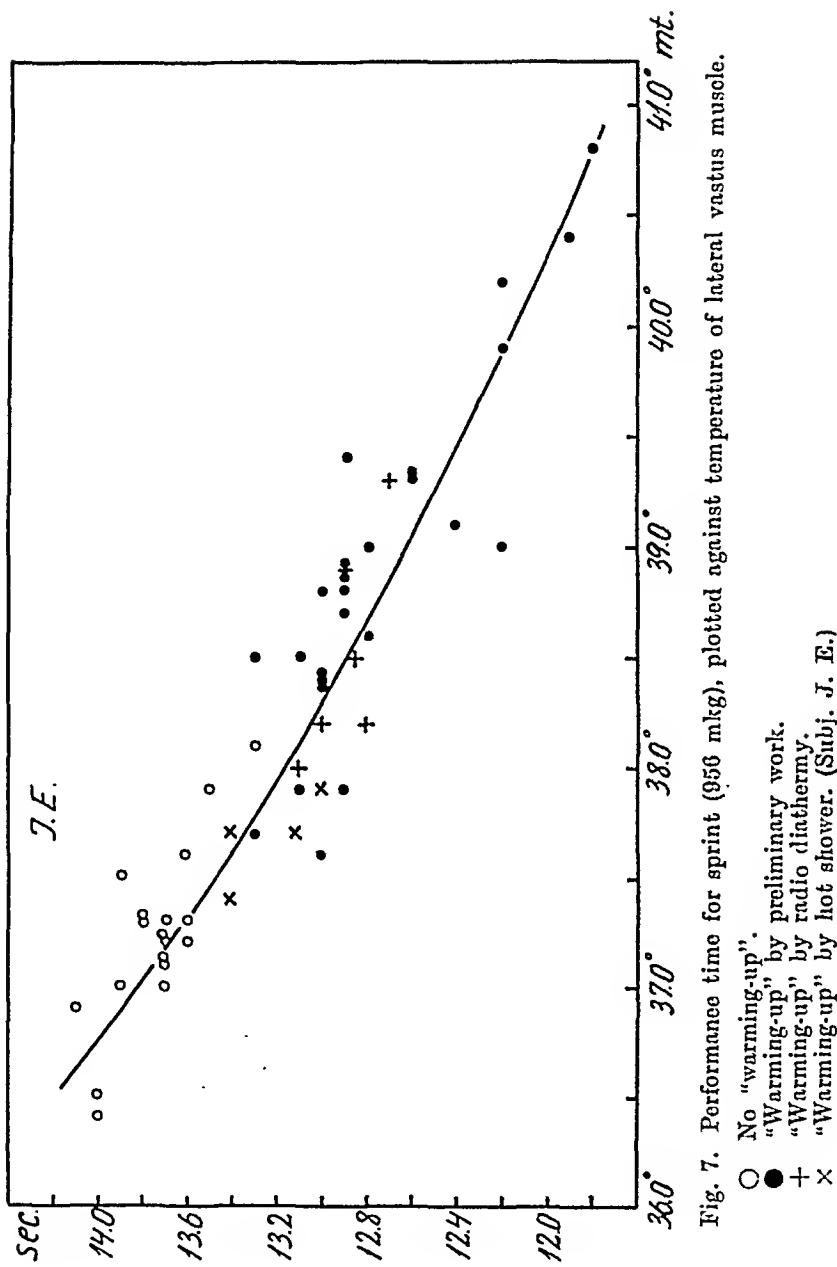


Fig. 7. Performance time for sprint (956 m/kg), plotted against temperature of internal vastus muscle.

○ No "warming-up".
 ● "Warming-up" by preliminary work.
 + "Warming-up" by radio diathermy.
 × "Warming-up" by hot shower. (Subj. J. E.)

dependent of whether the temperature has been achieved by active muscular work, or by passive warming by radio diathermy or hot baths.

The standard performances used in the above experiments for testing the effect of a warming-up have both consisted of several movements. It is necessary to see whether the effect of a higher

Table 7.

Subj.	"Cold"					"Warm"				
	Rectal temp.	Calf temp.	Arm temp.	Leg push	Arm pull	Rectal temp.	Calf temp.	Arm temp.	Leg push	Arm pull
J. E. . .	37.1°	36.1°	36.0°	178 kg	9.4 mkg	37.9°	38.1°	36.1°	201 kg	9.2 mkg
H. K. . .	36.8°	36.1°	35.9°	145 "	9.1 " "	37.7°	38.1°	35.2°	156 "	9.1 "

temperature manifests itself also in a peak effort of the type used in throwing or jumping. This was done by determining the maximum push that could be exerted by the muscles of the calf in a plantar flexion, and — in order to measure the maximum work performed by the arm muscles in a flexion of the elbow — by means of "HILL's fly-wheel". Both tests were performed, first after 30 minutes of rest, and then after 20 to 30 minutes of bicycle riding with a work intensity of 985 mkg/min. Rectal temperatures and the temperatures of the *soleus* muscle and the *biceps brachii* were measured just before the test. The results (averages of 7 experiments on each subject) are presented in table 7. (Subjects J. E. (data above) and K. H. (22 years, 175 cm, 63 kg).)

The table shows that an improvement is registered in the strength of the calf muscles, the temperature of which rises after the bicycling, not, however, in the performance of the elbow flexors, whose temperature does not increase. This latter in spite of the fact that there is a considerable rise in the rectal temperature.

In order to study the mechanism by which the improvement in work capacity may be brought about, determinations of the maximum oxygen-uptake during our long standard performance (450 revolutions) were made. In these experiments, the subject breathed through mouthpiece and valves during the entire period of work and the expired air was collected during the last 50 revolutions of the pedals and during the first 7 minutes of recovery. The total oxygen uptake during these 7 minutes of recovery allows a relative estimation of the magnitude of the oxygen debt acquired through the preceding work. Blood samples (capillary finger blood) were taken 3 and 6 minutes after cessation of work for estimation of the blood lactates. At this time of recovery the blood lactates were highest. These experiments were made only

on J. E., 5 experiments in the "cold" condition, and 5 in the "warm" condition after a preliminary work of 600 mkg/min. for 30 minutes. The results are presented in table 8.

Table 8
450 Revolutions.

Date	Time	Time for 50 last rev	O_2 -uptake during 50 rev. last rev (l/min)	"O ₂ -debt" 7 min. of recovery (l)	Blood lactate mg %
"Cold"					
8. Jan.....	4'32.5"	0.50"	3.995	5.456	—
12. Jan.....	4'32.3"	0.51"	3.964	5.895	135
17. Jan.....	4'34.2"	0.52"	3.916	7.140	133
22. Jan.....	4'33.5"	0.49"	4.021	7.190	132
30. Jan.....	4'34.1"	0.49"	4.141	6.544	137
Mean	4'37.8"	0.51"	4.023	7.552	133
"Warm"					
10. Jan.....	4'29.5"	0.48"	4.111	7.836	124
15. Jan.....	4'23.7"	0.47"	4.469	8.903	128
19. Jan.....	4'26.8"	0.46"	4.159	7.891	125
24. Jan.....	4'26.6"	0.46"	4.239	7.284	141
1. Feb.....	4'28.0"	0.465"	4.169	7.873	129
Mean	4'26.8"	0.47"	4.156	7.446	131

This table shows that the time required for the work has on an average decreased by 11 seconds or 4.0 per cent. The time for the last 50 turns has decreased relatively more, i. e. by 7.8 per cent. Correspondingly, the maximal O_2 -uptake during work is 0.103 liters per minute or 2.6 per cent higher in the "warm" series, and the "oxygen debt" has increased by 0.234 liters or 3.1 per cent. The blood lactate seems to be lower (3.0 %) in the "warm" series; the difference is so small, however, that it lies within the accuracy of the method, and other determinations of the blood lactate on subjects H. A., J. E., K. G. and F. J. after the performances referred to in table 1 show that the blood lactate is practically identical in the two conditions (table 9).

In the experiments on J. E. (table 8) the pulse rate was counted during the last phase of work. It varied in both series between 200 and 216, and no systematic difference between the two series could be detected.

Table 9.

Aver. Blood Lactates Corresp. to Work of 450 Rev. in Table 1.

Subj	"Cold" mg %	"Warm" mg %	Decrease or in- crease (%)
H. A.	128	131	+ 2.3
J. E.	158	160	+ 1.3
K. G.	176	173	- 1.7
F. J.	154	163	+ 5.8

Conclusions and Discussion.

The conclusions to be drawn from the experiments described on the preceding pages are: A higher temperature in the working organism facilitates the performance of work. This is clearly demonstrated for work consisting in a peak effort, as well as in work lasting 12 to 15 seconds, and in performances of longer duration (4 to 5 minutes). It has further been demonstrated that not only the active warming of the organism through a preliminary work, but also a passive heating of the organism by radio diathermy or by hot water, has a beneficial effect on the output of work, corresponding to the temperature it involves. From this it seems justifiable to conclude that the beneficial effect noted is preponderantly a *temperature* effect, and that other effects of the preliminary work (circulatory, respiratory, hormonal etc.) may be left out of consideration in this connection.

The effect seems to be almost proportional to the temperature, as figs. 6 and 7 show. From fig. 6 it is impossible to tell whether it is the rectal temperature (representing, presumably, the mean temperature of the arterial blood) or the local muscle temperature that is directly responsible for the improvement in work capacity. Certain observations seem, however, to indicate that it is the muscle temperature. These observations are: After a preliminary work of short duration (5 min.), the rectal temperature has increased only by 0.1 to 0.2° C. The effect on a subsequent performance is, however, considerable, closely related to the fact that the muscle temperature has increased considerably (table 6 and fig. 5). A prolongation of the preliminary work up to 30 minutes brings about a further increase of the rectal temperature of 0.5 to 0.9° C. This relatively large increase is not accompanied by a corresponding increase in performance, but by a rather moderate

improvement of such a value as might be expected from the concomitant increase in muscle temperature.

As further support of this assumption may be mentioned the experiments tabulated in table 7: In the "warm" condition the rectal temperature has in this case increased by about 0.8° C., but nevertheless the output of work by the elbow flexors is not larger, corresponding to the fact that their temperature has not increased, whereas the leg muscles could develop a considerably higher tension, in accordance with the higher temperature measured here.

Roughly spoken, an increase of the *muscle temperature* alone (table 6) improves the performance considerably, whereas an increase of the *rectal temperature* alone (table 7) seems to have but slight effect on the kind of work here investigated. The close dependence of work capacity on muscle temperature is also indicated by the curves of figs. 5 and 7. In a graph showing work capacity (performance time) plotted against *rectal temperature*, the values from experiments with short duration of preliminary work will fall outside the general curve for reasons mentioned above.

From a teleological point of view it looks rather natural that in an exercise of shorter duration the rapidly increasing muscle temperature plays a more important rôle than does the slowly changing rectal temperature. In exercises of longer duration (30 minutes and more) the fact may be different; anyway the accurately regulated rectal temperature during long exercises (NIELSEN (1938)) seems to make this assumption very reasonable.

The significance of a higher rectal temperature, i. e. a higher temperature of the circulating blood, may be sought in the fact, that a higher blood temperature allows the local muscle temperature to be maintained at a higher level than would otherwise be possible. The actual determinations of the muscle temperature show that the latter may vary during the work: e. g. in fig. 4 it is slowly increasing, very nearly corresponding to the concomitant increase in rectal temperature; but in fig. 5 the muscle temperature seems to be independent of the steadily increasing rectal temperature, and still other experiments, not to be published here, show that the muscle temperature after the initial rise may even fall somewhat, although the rectal temperature still increases. The muscle temperature is presumably rather susceptible to changes in skin temperature, so that for instance the outbreak of sweat-

ing may influence the temperature of the underlying muscles considerably. A higher temperature of the abundant blood flow irrigating the working muscles will in all cases permit them to maintain a higher and more adequate temperature. The accurate regulation of the blood temperature during work (NIELSEN (1938)) thus becomes a highly appropriate mechanism by which to improve the capacity for work.

A further beneficial effect of a higher rectal temperature (i. e. mean temperature of the arterial blood) may be looked for elsewhere, e. g. in the nervous system. An attempt to demonstrate such an effect was made by determining the reaction time eye-hand in the "cold" and "warm" condition. The determinations were made on our four main subjects and the average results came out as shown in table 10.

Table 10.
Reaction Times.

Subj.	"Cold"	"Warm"
H. A.	204 ± 5 msec.	190 ± 4 msec.
J. E.	188 ± 8 "	195 ± 10 "
K. G.	196 ± 6 "	205 ± 4 "
F. J.	189 ± 4 "	175 ± 3 "

The results are not conclusive, as in two subjects (H. A. and F. J.) there was a beneficial effect of the higher temperature, whereas J. E. and K. G. apparently showed the opposite. It may be mentioned, though, that the single determinations (18 of each kind) in both H. A. and J. E. scattered rather little, whereas in J. E. and K. G. the individual determinations (15 of each kind) varied somewhat more.

A beneficial effect of a higher blood temperature during work on other organs, e. g. heart or liver, seems quite possible, only we are not in a position to demonstrate it.

The ways in which the *local* temperature of the muscles may influence the ability to perform work is also open to discussion. The effect of the higher temperature may be assumed to be either of a *mechanical* or of a *chemical* nature.

Mechanically, a higher temperature of the working muscles may be of advantage by influencing the viscous and elastic properties of the muscles in such a way that at the same production of energy more external work could be performed. Viscosity de-

creases with increasing temperature, and it is quite possible that when temperature increases, less energy may be lost in overcoming viscous resistance in swift-moving muscles, so that in this way more energy may be utilized for the performance of external work. The elastic properties of muscle fibres have been studied by BUCHTHAL *et al.* (1944), who found that stiffness during contraction decreases with increasing temperature, that dynamic shortening increases somewhat, but that the height of extra-tension developed during contraction in frog muscles is hardly affected by even large changes in temperature (6° to 23°). Judging from these results, the beneficial effect of a higher muscle temperature can hardly be assumed to be the effect of temperature on the elastic properties of the muscles.

Chemically, a higher temperature may benefit muscular performances by accelerating the liberation of energy in the muscles. If the aerobic processes in muscles are accelerated, one might expect to find a higher maximal O_2 -uptake in the "warm" condition than in the "cold" condition. An indication of this is given by the data of table 8, showing that on an average the O_2 -uptake during the last 50 revolutions in the "warm" condition is increased by 0.1 l/min. or 2.6 per cent. When, however, the oxygen necessary for actually performing these revolutions is calculated it is found to be on an average 2.047 l in the "cold" condition and only 1.931 l in the "warm", a difference of 0.115 l, suggesting a reduction in the internal resistances.

The higher temperature, therefore, may quite well have increased the intensity of the aerobic processes in the muscles. Similar effects on the maximal O_2 -uptake have been observed by NIELSEN and HANSEN (1937), who found that after heavy preliminary work a subject could take up more oxygen in a final spurt than when the spurt had been begun from a resting condition. They were inclined to ascribe this effect to the increased circulation reached in the preliminary work. In our case such an effect can be disregarded as the subject in all cases began work after a pause of several minutes. (The final pulse rate reached was also practically the same: about 210.)

Not only the oxidative processes in the muscles but also the anaerobic must be assumed to be furthered by a higher temperature. We could, however, not demonstrate any changes in the formation of lactic acid (table 9), and the slight increase in the "oxygen debt" (i. e. the total oxygen intake in 7 minutes of re-

covery) (table 8) in the "warm" condition (0.234 l) is so small that the effect of the higher body temperature on the basal metabolism alone may explain the whole increase. But, on the other hand, the improvement in the "sprint" and in the "push" can hardly be explained without the assumption of a temperature effect on the anaerobic processes (and on the intramuscular resistances).

Of special interest for this discussion is the effect of a higher temperature on the dissociation curve of haemoglobin. According to BARCROFT and KING (1910), haemoglobin at an oxygen tension of 30 mm Hg gives up almost twice as much oxygen at 41° as at 36°, and the oxygen dissociates from haemoglobin about twice as rapidly. A corresponding effect of temperature on the dissociation curve of myoglobin has been demonstrated by THEORELL (1934) and although the temperature dependency is somewhat smaller than in haemoglobin, a beneficial effect on the transference of oxygen from blood to the intracellular oxydative systems may well be assumed.

It seems therefore quite reasonable to conclude that the beneficial effect of an increased temperature on the ability to perform muscular work is due not only to the effect of temperature on the chemical processes in the muscles, presumably both the anaerobic and the aerobic, but also to a mechanical factor (decreased viscosity).

Summary.

It is a well established fact that the body temperature increases during muscular exercise, and that the increase is regulated (NIELSEN, 1938). It was the purpose of this paper to study the effect of the increased body temperature on the performance of maximum work, viz. a work performance of short duration (12 to 15 seconds) and a work performance lasting 4 to 5 minutes. The effect on a peak effort (a "push" or a "pull") was also studied.

The experiments showed:

- 1) A given amount of work could be performed better — i. e. in a shorter time — when the organism was warmed up by a preliminary work. Also a greater muscular tension could be developed when "warmed up" than when not.
- 2) A passive warming up — e. g. by means of radio diathermy or by hot baths — also increased the capacity for work.
- 3) Massage had no beneficial effect on the performance

4) The harder the preliminary work was, the higher rose the temperature and the better was the performance (only demonstrated in the work of shorter duration (12 to 15 sec.)).

5) The increased ability to perform hard work is closely correlated to the temperature of the working muscles.

6) The maximum oxygen uptake is slightly higher when the organism is warmed up than when this is not the case, but the oxygen necessary for a certain amount of work is reduced.

From these results it is concluded that a higher temperature in the muscles benefits the ability to perform work by accelerating the chemical processes in the muscles, probably also by decreasing the intramuscular viscous resistance. The accurately regulated higher rectal temperature in work (NIELSEN) allows the muscles to obtain a higher temperature during work than would otherwise be possible.

The expenses of this work have been covered by grants from the *Frk. P. A. Brandts Legat* Foundation to which we are much indebted.

References.

BARCROFT, J. and W. O. R. KING, J. Physiol. 1909—10. 39. 374.
 BENEDICT, F. G. and J. F. SNELL, Pflüg. Arch. ges. Physiol. 1902. 90. 33.
 BUCHTHAL, F. and E. KAISER, Acta physiol. scand. 1944. 8. 38.
 BUCHTHAL, F., E. KAISER and G. G. KNAPPEIS, Ibidem. 1944. 8. 16.
 BUCHTHAL, F., P. HÖNCKE and J. LINDHARD, Ibidem. 1945. 8. 230.
 CHRISTENSEN, E. H., Arbeitsphysiologie 1931. 4. 154.
 EDWARDS, H. T., J. biol. Chem. 1938. 125. 571.
 HANSEN, T. E. and J. LINDHARD, J. Physiol. 1923. 57. 287.
 HILL, L. and M. FLACK, Ibidem 1907. 36. Proc. XI.
 JÜRGENSEN, Die Körperwärme des ges. Menschen. Leipzig 1873.
 LINDHARD, J., Medd. om Grönland. 1910. 44. 1.
 NIELSEN, M. and O. HANSEN, Skand. Arch. Physiol. 1937. 76. 37.
 NIELSEN, M., Ibidem. 1938. 79. 193.
 PEMBREY, M. S. and B. A. NICOL, J. Physiol. 1898. 23. 386.
 THEORELL, H., Biochem. Z. 1934. 268. 73.
 ZUNTZ, N. and SCHUMBURG, Physiologie des Marsches. Berlin 1901.
 ZUNTZ, N., A. LOEWY, F. MÜLLER and W. CASPARI, Höhenklima und Bergwanderungen. Berlin 1906.

From Universitetets Biokemiske Institut, Copenhagen.

On Serum Choline Esterase Activity in Experimental Liver Injury.

By

GUNNAR STEENSHOLT and HELGE VENNDT.

Received 5 April 1945.

The enzyme choline esterase has been very extensively studied during recent years, both by biochemists and physicians, on account of its important functions in the nervous system. A very large experimental material has thereby been brought together. However, our knowledge of the enzyme is still very incomplete, and a number of fundamental questions still await their solution. Among these is the very important problem as to the place of origin of the enzyme in the animal organism. So far, the question seems to have been approached only from the clinical side. A complete account of all the work done up to now cannot be given here (for a summary see, for instance, FABER (1941)); much of it has been carried out with rather crude methods, and we shall therefore restrict ourselves to a consideration of only those investigations in which the determination of the serum choline esterase activity was made by the manometric method, since this seems to be by far the best available at the present time.

Thus, ANTOPOL, TUCHMAN and SCHIFRIN (1937, 1938) have reported an investigation of 500 patients with various diseases. Though their material shows very large variations, they always found particularly low serum choline esterase activity in patients with anemia and fibrilia, and especially with liver diseases. This feature was brought out much more clearly in the clinical material presented by McARDLE (1940). From an investigation of about 270 cases he concluded that diseases of the liver are

always accompanied by low serum choline esterase activity, and he even suggested the introduction of routine serum choline esterase activity determinations as a convenient test of liver function. FABER (1941) also presented a considerable clinical material, which showed uniformly the same characteristic feature of very low serum choline esterase activities in patients with liver diseases, and also in many cases of anemia and kidney and heart diseases. It was further found that low serum choline esterase activity was as a rule accompanied by a lowered amount of serum albumin. As the most probable explanation FABER suggests the liver cells as the common place of origin of both choline esterase and serum albumine, and, moreover, that there is a fairly constant ratio between the rates at which these two substances are excreted into the blood stream. FABER maintains that this assumption suffices to explain all the clinical observations.

So far, all investigations of these problems seem to have been confined to clinical work. We have therefore thought it worth while to study the variations of serum choline esterase activity in cases of experimental liver injury. The present paper is a report on the results of our work.

Experimental Part.

Methods of analysis. For the determination of serum choline esterase activity we have applied the manometric method of WARBURG, in the form developed by AMMON especially for this purpose. In our modification the procedure was as follows:

All measurements were carried out with a WARBURG equipment of ordinary construction. The volume of the reaction vessels was about 15 ml. Each vessel contained, in the main chamber, 2 ml bicarbonate Ringer containing 1 per cent serum. The side chamber contained 0.5 ml of a 3 per cent aqueous acetyl choline chloride solution. The vessels were filled with a mixture of 5 per cent CO₂ and 95 per cent N₂, and placed in a water bath of 37.5° C. After 15—20 minutes temperature equilibrium was reached, and the content of the side vessel was tipped into the main chamber. After another 5 minutes the readings were started and were taken every 10th minute. A thermobarometer was always applied in the familiar way. Under these conditions the liberation of CO₂ during the enzymic hydrolysis was found to proceed linearly with the time for a period considerably beyond 40 minutes. As a convenient measure of serum choline esterase activity we have used the amount of CO₂ liberated in 40 minutes, under the experimental conditions defined above. This quantity was determined graphically in

the way indicated in Fig. 1. All measurements were carried out in double and sometimes triple determinations. They usually agreed within 2—2.5 per cent.

Serum choline esterase is an enzyme of remarkable stability. In the literature one finds the statement that serum can be stored in the ice box for up to three weeks without measurable diminution of its activity, and this agrees well with our experience. In our work blood samples were never allowed to remain in the ice box for more than two weeks.

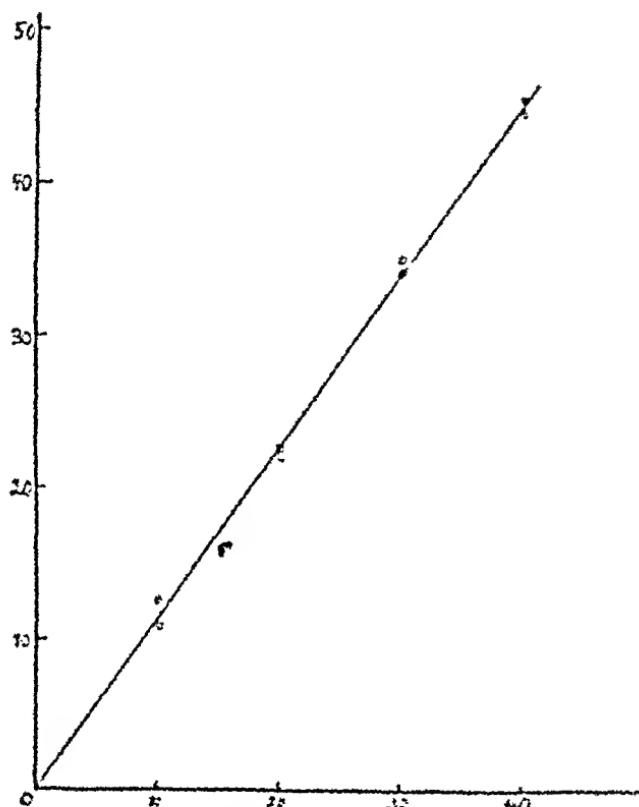


Fig. 1. Shows an example of the determination of serum choline esterase activity. Abscissa: Time in minutes; ordinate: $\text{cm}^3 \text{CO}_2$ evolved during the hydrolysis.

The determination of serum proteins were carried out by the well known methods described, for instance, in the textbook of PETERS and VAN SLYKE (1932). Likewise the icterus index (bilirubin index) was determined in the familiar way with the MARXSEN-WITT bilirubin colorimeter. All these measurements were carried out immediately after the blood had been drawn.

Experimental animals. Two dogs, A and B, were used. A was a male, of 25 kg body weight. B was a female, weighing 19 kg both were about 12—14 months old. They were kept on a diet of breast milk and water, sugar and liver patty, and in addition they received meat and bones once a week. The quantity of food offered was always in

excess of the actual demand of the animals. During the experimental period the animals seemed to lose appetite and probably decreased slightly in weight.

The chloroform was given per os mixed with 30—40 ml of mustard seed oil. No vomiting was observed. The blood samples were always drawn from the tarsal veins and always before the chloroform was given.

Between each series of experiments the animals were allowed ample time for complete restitution.

Results. The following series of experiments were carried out.

In the first series each dog received daily 2.5 ml of chloroform for a period lasting about a week. The results are shown diagrammatically in Fig. 2. The serum choline esterase activity at the

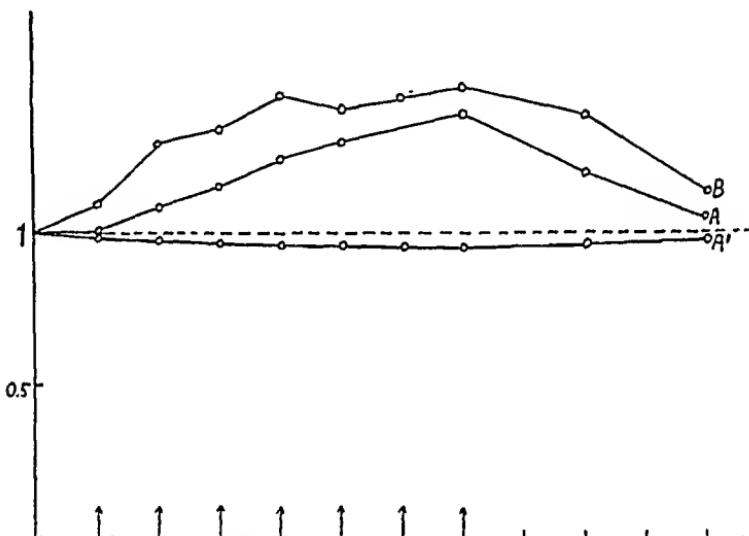


Fig. 2. The curves A and B give the variations in serum choline esterase activity; A' gives the variations in serum albumin. The abscissa axis is divided into 24-hours-intervals. The arrows denote the times at which chloroform was given. The serum albumin curve B' is left out since it is only very slightly different from A'.

beginning of the experiment was, for convenience, arbitrarily put equal to 1. No bilirubin determinations were carried out in this series.

In the next series a single large dose of 8 ml chloroform was given at the start of the experiment, and the serum choline esterase activity, the icterus index and the serum albumin were followed as described above. Fig. 3 a contains all the results. Here also the serum protein values at the beginning of the experiment were arbitrarily put equal to 1. Only the serum albumin

values are given, since they are of special interest for our investigation. The values obtained for the amount of total protein indicated a slight decrease; with a corresponding slight increase in serum globulin.

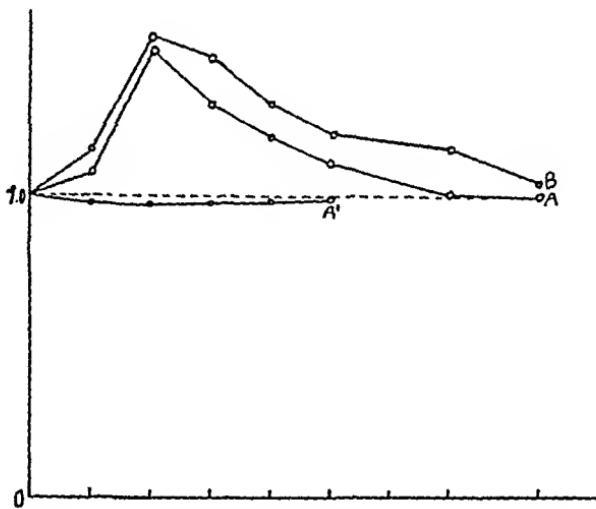


Fig. 3 a. All symbols have the same meaning as in Fig. 2. Abscissa divided into 24-hours-intervals.

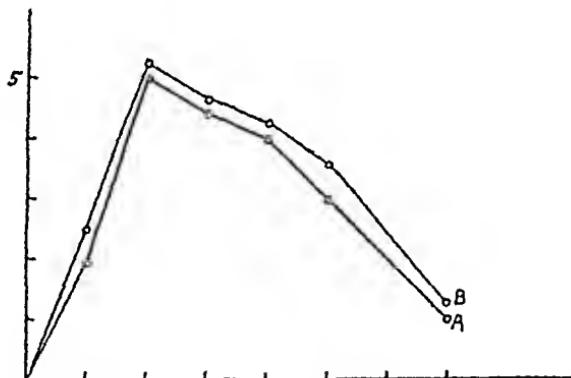


Fig. 3 b. Variations in bilirubin index during the experiment illustrated in Fig. 3 a.

Fig. 4 a presents in the same manner the results obtained on our last series of experiments. Here each dog received 4 ml chloroform at the times indicated in the figure.

A glance at the diagrams shows that in all our experiments the results were uniformly of the same general character, the qualitative nature of the reactions of the animals being the same in all experiments. We therefore limited our work to only two dogs.

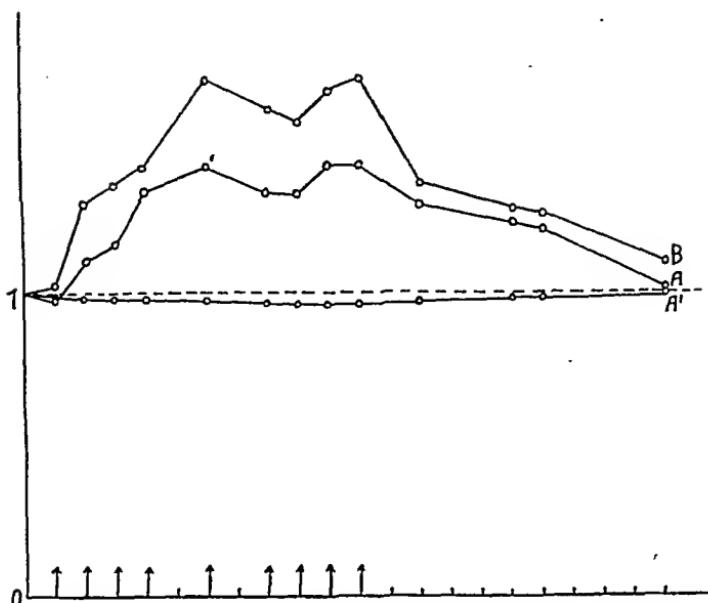


Fig. 4 a. All symbols, including the arrows, have the same meaning as in Fig. 2. The curve B' is left out for similar reasons as in Figs. 2 and 3. The abscissa is divided into 24-hours-intervals.

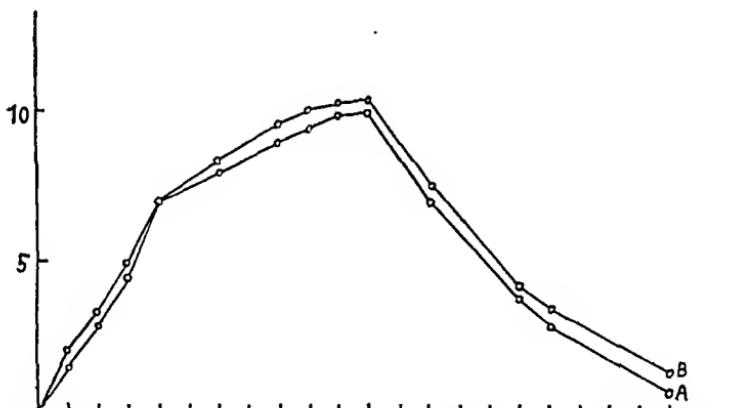


Fig. 4 b. Variations in bilirubin index during the experiment described in Fig. 4 a.

Discussion.

The striking feature of our results is the pronounced increase in serum choline esterase activity following upon administration of chloroform. Further we see that in contrast to the findings in clinical observations the rise in the serum choline esterase activity is accompanied by a simultaneous decrease in serum albumin.

A consideration of Figs. 2 and 3 shows that the curves for one single large initial dose of chloroform and the curves for con-

tinuous administration behave identically as regards the inter-relation of the variations in the factors investigated. In Fig. 4 a, however, the administration of chloroform has been interrupted on certain days, as indicated in the figure, and the curves therefore do not show the regular course of those in the Figs. 2 and 3. It appears from the diagram that an interruption in the administration of chloroform caused an immediate fall in serum choline esterase activity; when administration of the drug was resumed the enzyme activity rose again. No corresponding variations in the other factors could be found in our experiments, probably because they were too small to be detected with our methods of analysis.

The main result of the present experiments is, therefore, that in experimental liver injury by chloroform poisoning there is a marked increase in serum choline esterase activity accompanied by a simultaneous fall in serum albumin. As was pointed out above, this is quite contrary to the clinical findings, and seems therefore to throw doubt on the validity of some of the arguments hitherto brought forward in favour of the hypothesis of the liver as the place of origin of the serum choline esterase. However, we do not by any means maintain that this hypothesis has been refuted by our experiments. It must be borne in mind that chloroform poisoning is a very complex phenomenon, and although it is generally conceded that the liver is the organ mainly affected, other tissues may be influenced as well. On the other hand, it should not be forgotten that for similar reasons liver diseases are probably processes of equally high complexity. However, it may not be unsafe to conclude that the serum choline esterase activity is not unambiguously determined by liver function, and further, that the study of the variations of serum albumin does not afford any immediate or obvious clue to the problem under consideration. Much further work is needed to clear up these questions.

Summary.

It is found in experiments on dogs, that the serum choline esterase activity increases in experimental liver injury by chloroform poisoning. The simultaneous variations in serum albumin and icterus index are studied. The relation of the results to previous clinical work is discussed, and it is concluded that argu-

ments previously adduced in favour of the assumption that the liver is the place of origin of choline esterase, may need revision in certain respects.

References.

ANTOPOL, W., L. TUCHMAN, and A. SCHIFRIN, Proc. Soc. Exp. Biol. N. Y. 1937. 36. 46.
ANTOPOL, W., L. TUCHMAN, and A. SCHIFRIN, ibid. 1938. 38. 363.
FABER, M., Medical Thesis. 1941.
MCARDLE, B., Quart. J. Med. 1940. 9. 107.
PETERS, R., and D. D. VAN SLYKE, Quantitative Clinical Chemistry, London 1932. Vol. 2.

From the Biochemical Institute of the University, Aarhus,
Denmark.

The Influence of Adrenal Cortex on the Cholesterol Content in Rabbit Serum.

By

JØRGEN HOFFMEYER.

Received 19 April 1945.

In a previous paper (HOFFMEYER 1944) the cholesterol content in rabbit serum under normal and certain special conditions was investigated (anaemia, inanition, ether narcosis, surgical operations), and it was demonstrated that both daily bleeding with a decrease in haemoglobin below 55 per cent and moderate reduction of food intake is followed by hypercholesterolemia, whereas neither ether narcosis alone nor ether narcosis combined with surgical operations corresponding to extirpation and transplantation of adrenals seem to influence the serum cholesterol concentration.

In this paper it will be shown that adrenal cortex contains a factor which increases the cholesterol content in serum.

Material and Technique.

White rabbits were used for the experiments, and the experimental conditions were as previously reported (HOFFMEYER 1944).

Adrenalectomy is carried out by ventral laparotomy and in ether narcosis. The two adrenals are removed in two seances at an interval of 2 to 3 months. The operation lasts 15—20 minutes.

The transplantation is carried out with adrenals from young rabbits (500 to 1,500 g). 3 to 16 small adrenals (25 to 80 mg) almost cut through are transplanted into the abdominal wall of each animal in 1 seance. The transplants are placed in small pockets subeutaneously, intermuscularly and preperitoneally. The operation takes place in ether narcosis and lasts for about 15 minutes.

That the adrenal cortical hormone is really transferred by the transplantation technique employed, appears from the experiments mentioned below on the influence of transplants on the survival of rabbits, which have been properly adrenalectomized (Table 1). In these animals the adrenals were removed at an interval of 3 days, and the transplanta-

tion of small adrenals was undertaken in the same seance as the first adrenalectomy.

Table 1.

Survival of Rabbits after Bilateral Adrenalectomy and Adrenal Transplantation.

Rabbit No.	Sex	Time of survival	Weight in g at		Death	Transplanted adrenals		Adrenal tissue left or accessory adrenals		
			Adrenal-ectomies			Number	Total weight (approximate mg)			
			1st	2nd						
377	♂	30 Days (killed)	2720	2640	2620	3	240	—		
388	♂	10 Days	1660	1435	1030	3	240	—		
400	♂	30 Days (killed)	1950	1810	1810	5	400	—		
404	♀	11 Days	2370	2160	1700	10	250	—		
407	♂	30 Days (killed)	2120	1900	1660	5	400	—		
<i>Controls</i>										
391	♀	5½ Days	1920	1560	1340	—	—	—		
392	♂	10 Hours	2000	1800	—	—	—	—		
394	♂	10 Hours	1900	1765	—	—	—	—		
396	♂	4 Days	1920	1640	1360	—	—	—		
397	♂	13 Hours	2080	1880	—	—	—	—		
398	♂	10 Hours	1950	1870	—	—	—	+		
399	♂	12 Hours	1970	1700	—	—	—	—		
406	♂	15 Hours	1980	1800	—	—	—	—		

In all animals a thorough post mortem examination was made concerning the presence of accessory adrenals or adrenal tissue left at the places of extirpation.

As will be seen from Table 1, the survival time of the 5 rabbits, on which both adrenals were removed and into which new adrenals were transplanted, was 10 days or more, whereas the survival time of the 8 control animals, which were subjected to bilateral adrenalectomy alone was 5½ days or less. The conclusion appears to be that the adrenal transplants seem to possess a considerable adrenal cortical function possibly owing to preformed hormone in the transplants.

The preparations used for injection experiments were 1) adrenal extract, Ecortan ("MCO") prepared from whole adrenals according to SWINGLE and PFIFFNER's method; this preparation was stated to have such a strength that a daily injection of $\frac{1}{4}$ cc was able to keep 50 per cent of properly adrenalectomized rats alive, 2) adrenal extract (called preparation X by the author) prepared by "MCO" in exactly the same manner as Ecortan, but for some unknown reason unable to keep adrenalectomized rats alive, 3) synthetic cortical preparation, Ocriten (BAYER), which was stated to contain 5 mg desoxycorticosteronacetate (D. C. A.) dissolved in 1 cc of sesame oil.

The cholesterol analyses were carried out according to BRUN's method (1935). The haemoglobin concentration in blood was determined by the siccata-method (HESSE and TRIER 1937), the cell volume of the blood

with C. M. VAN ALLEN's method. The inaccuracy of all three methods was less than 2 per cent.

I. The Influence of Adrenal Transplantation on the Cholesterol Content in Serum.

Reports on the influence of adrenal transplants on the serum cholesterol have not previously been given.

In what follows (Table 2) are presented the results of such investigations in the days immediately following the transplantation.

Table 2.
The Serum Cholesterol after Transplantation of Adrenals.

	Before transpl. Days			After transplantation Days								
	3	2	1	1	2	3	4	5	6	7	8	9
Rabbit No. 362.												
Weight in g....	2130	2110	2110	1930	1975	1990	1980	1980	1970			
Food intake (Kohlrabi) in g....	400	320	290	180	250	280	300	290				
Hæmoglobin %.	70	60	61	60	60	56	55	55	54			
Totalchol. mg%.	35.6	32.9	33.7	30.1	37.9	37.4	44.5	54.2	49.7			
Rabbit No. 353.												
Weight in g....	2040	2050	2100	2000	2030	1990	2010	2000	1900	1975		
Food intake (Kohlrabi) in g....	400	400	400	400	400	400	400	400	400	400		
Hæmoglobin %.	80	72	—	75	60	65	60	58	55	50		
Totalchol. mg%.	16.5	20.0	20.2	18.2	37.5	26.7	44.5	34.1	29.3	23.4		
Rabbit No. 410.												
Weight in g....	1990	2100	2060	2000	2110	2150	2050	2040	2040	2015	2030	2110
Food intake (Kohlrabi) in g....	500	500	250	450	370	500	420	500	460	500	500	500
Hæmoglobin %.	67	68	70	70	60	55	55	58	55	55	50	50
Cell volume %.	26	26	26	25	23	22	23	23	22	22	22	22
Totalchol. mg%.	22.3	23.2	19.8	32.0	33.1	32.0		45.9		47.3		40.3
Rabbit No. 411.												
Weight in g....	1850	1790	1750	1630	1700	1750	1735	1800	1730	1700	1770	1775
Food intake (Kohlrabi) in g....	500	500	50	350	375	450	500	500	500	500	500	500
Hæmoglobin %.	80	70	68	65	65	63	62	60	60	62	60	60
Cell volume %.	30	29	28	27	25	25	25	25	24	26	25	25
Totalchol. mg%.	17.0	16.6	15.1	23.1	24.6	26.7	21.5	19.4	22.9	23.5	22.2	20.7

In rabbit No. 362 and 353 16 small adrenals (approximate total weight: 400 mg) were transplanted, in rabbit No. 410 and 411 3 big adrenals (approximate total weight: 240 mg) were transplanted.

It will be seen from the table that after the transplantation a hypercholesterolemia of somewhat varying strength appears. It begins on the 1st to the 4th day, reaching a maximum on the 2nd to the 7th day, and has practically disappeared 7 to 8 days after the transplantation. The alterations in haemoglobin percentage, cell volume and intake of food show that it is possible to exclude unspecific causes of this hypercholesterolemia, and the hypercholesterolemia must thus have been provoked by the tissue transplanted. That the hypercholesterolemia is not due to the content of adrenal marrow or adrenalin in the transplants appears from Table 3, which contains the results of experiments with separate transplantation of cortex and marrow and of an experiment with daily injection of adrenalin. Only in the case of transplantation of adrenal cortical tissue was a hypercholesterolemia demonstrated.

Table 3.

The Serum Cholesterol after Separate Transplantation of Adrenal Cortex and Marrow and after Daily Injection of Adrenalin.

	Before transpl. Days			After transplantation Days					
	3	2	1	1	2	3	4	5	6
Rabbit No. 323.									
Weight in g	1610	1630	1600	1590	1505	1530	1540	1555	1520
Food intake (Kohlrabi) in g ..	395	400	160	250	310	390	325	400	
Haemoglobin %	85	83	80	80	72	70	62	55	
Totalchol. mg %	55.6	60.0	62.0	104	94.1	89.3	78.1	65.5	62.8
Rabbit No. 366.									
Weight in g	1910	1930	1940	1870	1885	1915	1840	1885	1870
Food intake (Kohlrabi) in g ..	400	400	400	400	390	390	400	400	
Haemoglobin %	99	95	91	80	80	78	76	65	60
Totalchol. mg %	45.3	49.3	42.4	46.6	42.6	38.1	46.3	51.9	51.3

In rabbit No. 323 the cortical tissue of 16 small adrenals was transplanted. In rabbit No. 366 the marrow of 16 small adrenals was transplanted.

	Before inject. Days			During daily injections of $\frac{1}{2}$ mg adrenalin intramuscularly Days						
	3	2	1	1	2	3	4	5	6	7
Rabbit No. 312.										
Weight in g	2830	2800	2800	2780	2760	2760	2710	2650	2650	2640
Food intake (Kohlrabi) in g ..	400	400	400	400	350	400	400	400	400	400
Haemoglobin %	75	75	75	75	80	82	76	70	65	74
Totalchol. mg %	16.2	21.4	16.0	16.0	18.6	19.7	22.9	16.3	15.1	17.2

II. The Influence on the Cholesterol Content in Serum of Adrenal Cortical Preparations.

It has not been possible to investigate properly the influence of cortical extracts on serum cholesterol until SWINGLE and PFIFFNER in 1930 had prepared the first really useable cortical extract. The reports in the literature of the subject on the influence of the SWINGLE and PFIFFNER preparations on serum cholesterol are, however, scarce, and these works have led to widely disagreeing results; thus some authors (THADDEA and FASSHAUER 1936, MEDVEI 1935, BAUER and BUTTU 1932) have found a cholesterol-decreasing effect, others (MARANON and COLLAZO 1935, BERNHARDT and SIMPSON 1932) a cholesterol-increasing effect, and still others

Table 4.

The Serum Cholesterol after Daily Repeated Injections of Adrenal Cortical Preparations.

	Before inject. Days			During injections Days						
	3	2	1	1	2	3	4	5	6	7
Rabbit No. 312.										
Weight in g	2500					2465			2400	
Hæmoglobin %		80							55	
Injection of Ecortan ml ..				2.5	2.5	2.5	2.5	2.5	2.5	2.5
Totalchol. mg %	22.1	21.8	35.6	34.0	43.1	49.2	64.6	56.5	63.7	
Rabbit No. 353.										
Weight in g	2350	2285	2340	2310	2280	2270	2220			
Food intake (Kohlrabi) in g	400	350	330	300	400	400				
Hæmoglobin %	88	85	70	70	68	65	60			
Cell volume %	31	30	28	29	27	26	23			
Injection of Ecortan ml ..				4.6	4.6	4.6	4.6			
Totalchol. mg %	32.2	34.5	34.2	40.3	52.5	43.5	49.1			
Rabbit No. 415.										
Weight in g	1860		1880	1800	1715	1750	1800	1780	1780	
Food intake (Kohlrabi) in g			500	360	450	500	430	325	480	
Hæmoglobin %	82		80	80	78	75	60	55	50	
Cell volume %	23		23	23	22	21	22	20	20	
Injection of Ocriten ml ..				1	1	1	1	1	1	
Totalchol. mg %	28.6	26.4	30.7	32.3	27.0	25.0	28.0	26.0	34.6	
Rabbit No. 332.										
Weight in g	2350	2300	2250	2230	2350	2310	2375	2300		
Food intake (Kohlrabi) in g	400	400	400	400	380	340	400	400		
Hæmoglobin %	85	85	83	74	60	60	50	65		
Injection of Ocriten ml ..					1	1	1	1		
Totalchol. mg %	42.2	43.5	43.5	37.4	42.3	45.4	40.9	40.6		

(HARROP, PFIFFNER and WEINSTEIN 1931) no effect at all of SWINGLE and PFIFFNER preparations on the serum cholesterol.

The author has investigated the question and carried out experiments with 2 adrenal extract preparations (Ecortan, preparation X) and a synthetic cortical hormone preparation (Ocriten). After having shown in preliminary experiments that single injections of Ecortan and Ocriteren respectively did not involve alterations of the serum cholesterol content after a few hours, investigations were carried out with daily injections during one week.

The results of these experiments are given in Table 4, from which it appears that whereas daily injection of Ecortan causes hypercholesterolemia, Ocriteren is without such an effect.

It ought to be mentioned that the hypercholesterolemia was provoked by Ecortan doses (1 to 2 cc per kg body weight) which — measured in relation to the life-sustaining effect — were much smaller than the doses of Ocriteren employed. The hypercholesterolemia appeared 1 day after the beginning of treatment with injections, reaching a maximum in 2 to 5 days, and kept fairly constant as long as the injections were continued.

The above-mentioned results could be verified with the other cortical extract (preparation X) (Table 5), which for some reason or other was not able to keep adrenalectomized rats alive.

Table 5.

*The Serum Cholesterol after Daily Repeated Injections
of Preparation X.*

	Before inject. Days			During injections Days					
	3	2	1	1	2	3	4	5	6
Rabbit No. 424.									
Weight in g	1650	1600	1660	1700	1620	1640	1640	1670	1650
Food intake (Kohlrabi) in g ..	430	430	385	500	455	410	360	440	
Hæmoglobin %	85	85	84	74	68	65	63	60	55
Cell volume %	30	29	29	26	26	25	26	26	25
Injection of Prep. X ml				2.5	2.5	2.5	2.5	2.5	2.5
Totalchol. mg %	34.5	35.0	32.3	38.8	46.8	46.3	48.8	55.8	64.4
Rabbit No. 425:									
Weight in g	2260	2240	2300	2220	2200	2280	2250	2280	2275
Food intake (Kohlrabi) in g ..	500	500	500	450	450	375	500	400	
Hæmoglobin %	85	75	70	65	65	—	60	58	
Cell volume %	32	32	30	29	27	28	27	26	
Injection of Prep. X ml				6.6	6.6	6.6	6.6	6.6	6.6
Totalchol. mg %	29.8	31.3	27.6	32.8	40.3	39.7	40.2	42.8	42.9

In consequence of experiments undertaken — as unspecific causes seem to be excluded — it must be supposed that Ecortan contains a substance which increases the cholesterol content in serum, and that this substance is not identical with the life-preserving cortical hormone desoxycorticosteronacetate.

III. The Influence of Adrenalectomy on the Cholesterol Content in Serum.

Several authors (CHAUFFARD and GRIGAUT 1914, ROTHSCHILD 1915, LANDAU and McNEE 1914, HUECK 1914, THADDEA and FASSHAUER 1936) state that adrenalectomy is followed by hypercholesterolemia, whereas other authors (BAUMANN and HOLLY 1923, RANDLES and KNUDSON 1928, SILBERSTEIN, WACHSTEIN and GOTTDENKER 1932) found no change in serum cholesterol after adrenalectomy.

Table 6.

The Serum Cholesterol after Bilateral Adrenalectomy.

	Before adrenal- ectomy Days			After adrenalectomy Days								
	3	2	1	1	2	3	4	5	6	7	8	9
Rabbit No. 373.												
1st Adrenal- ectomy												
Weight in g.....	2050	2040	2050	2150	2000	1950	2010	1900	2000	2070	2020	2070
Food intake (Kohlrabi) in g.....	445	500	170	460	450	550	500	500	500	500	500	500
0.9 % NaCl ml..	0	0	0	50	70	75	0	0	0	0	0	0
Hæmoglobin %..	85	82	85	75	63	60	60	62	55	55	58	65
Cell volume %..	35	35	33	30	29	29	29	30	26	25	28	30
Totalchol. mg%..	33.4	41.6	39.6	39.8	34.5	35.4	32.3	33.8	35.1	37.7	38.6	35.8
2nd Adrenal- ectomy												
Weight in g.....	2450	2400	2375	2360	2370	2375	2260	2220	2190	2190	2120	2190
Food intake (Kohlrabi) in g.....	500	500	0	0	50	50	200	310	500	280	500	500
0.9 % NaCl ml..	0	0	0	100	100	50	75	190	0	90	55	58
Hæmoglobin %..	90	88	85	85	82	80	75	63	60	60	55	58
Cell volume %..	34	32	28	29	28	28	25	22	21	21	19	20
Totalchol. mg%..	19.8	19.5	20.8	21.2	27.6	27.1	24.0	23.9	45.2	48.7	43.5	

	Before adrenalectomy Days			After adrenalectomy Days								
	3	2	1	1	2	3	4	5	6	7	8	9
Rabbit No. 375.												
<i>1st Adrenal-ectomy</i>												
Weight in g.....	2505	2465	2375	2350	2330	2300	2350	2280	2370	2360	2380	2430
Food intake (Kohlrabi) {.....	500	500	400	465	450	500	500	500	500	500	500	500
in g												
0.9 % NaCl ml..	0	0	0	50	90	75	0	0	0	0	0	0
Hæmoglobin %..	85	80	80	78	60	60	58	55	55	58	55	60
Cell volume % ..	34	33	31	30	29	29	29	30	29	27	27	27
Totalchol. mg% ..	21.4	27.8	29.8	29.8	26.3	26.0	27.5	29.6	33.4	33.1	33.4	32.4
<i>2nd Adrenal-ectomy</i>												
Weight in g.....	2610	2630	2595	2540	2500	2420	2335	2250	2270	2270	2260	2350
Food intake (Kohlrabi) {.....	500	500	0	0	0	40	65	65	420	420	420	500
in g												
0.9 % NaCl ml..	0	0	0	20	50	50	50	75	25	0	0	0
Hæmoglobin %..	78	75	75	72	68	65	68	70	70	68	65	60
Cell volume % ..	30	29	26	24	22	22	22	24	22	24	22	22
Totalchol. mg% ..	21.6	20.6	23.1	38.3	40.1	48.9	55.3	63.6	68.4	68.8	115	105
Rabbit No. 382.												
<i>1st Adrenal-ectomy</i>												
Weight in g.....	2565	2565	2580	2550	2460	2445	2475	2480	2490	2450	2360	
Food intake (Kohlrabi) {.....	500	500	400	365	450	500	500	500	500	500	500	
in g												
0.9 % NaCl ml..	0	0	0	75	60	75	75	100	0	0	0	
Hæmoglobin %..	80	82	82	78	75	70	65	65	70	65	62	
Cell volume % ..	34	33	32	26	25	26	26	24	24	26	27	
Totalchol. mg% ..	28.9	30.5	33.7	39.9	29.7	29.2	57.9	57.4	44.6	53.8	48.0	
<i>2nd Adrenal-ectomy</i>												
Weight in g.....	2860	2810	2720	2750	2725	2640	2570	2540	2625	2625	2620	
Food intake (Kohlrabi) {.....	500	500	0	100	170	270	385	385	430	450		
in g												
0.9 % NaCl ml..	0	0	0	100	100	75	25	85	100	0	0	
Hæmoglobin %..	72	75	80	78	65	65	60	62	60	58	55	
Cell volume % ..	25	26	30	30	25	26	25	25	24	23	26	
Totalchol. mg% ..	22.4	26.9	22.9	27.3	26.5	32.4	35.1	56.3	57.9	61.6	42.3	

All 3 rabbits survived the adrenalectomies. The post mortem examination: small cortical rests on the places of extirpation or small accessory adrenals were demonstrated in all three cases.

It appears, however, from the literature referred to, that most of these investigations have been carried out not considering that several previously mentioned factors (anaemia, haemoconcentration, inanition) may have interfered with the experimental results.

In Table 6, given below, are presented the results of some investigations in which haemoglobin percentage, cell volume, food intake and body weight of the animals have been carefully followed, and simultaneously it has been tried to avoid any anaemia of importance, and by addition of 0.9 per cent NaCl-solution to the drinking-water an attempt has been made to sustain the appetite and food-intake of the animals in connection with the adrenalectomy. The two adrenals were removed at an interval of 2 to 3 months.

As will be seen the table includes 3 rabbits for which the cholesterol level has been examined after successive removal of both adrenals. In all cases no rise or only a small increase in the serum cholesterol values after the 1st adrenalectomy was found, but after the 2nd adrenalectomy hypercholesterolemia appeared; this hypercholesterolemia, however, in 2 of the 3 animals did not begin until after some days, i. e. somewhat delayed compared with the findings of earlier authors. It appears from the table that the hypercholesterolemia observed could not be explained as a result of anaemia or haemoconcentration, but very likely as a result of inanition, as it was not possible — in spite of the addition of NaCl — to avoid a quite considerable decrease of food intake and body weight after the 2nd adrenalectomy.

Discussion.

The statements reported concerning the influence of adrenal cortex on the cholesterol concentration in serum are so contradictory that new investigations in this field are desirable.

The results presented in this paper indicate, that transplantation of adrenals causes hypercholesterolemia, and that this hypercholesterolemia is due to a hyperfunction of the adrenal cortical tissue through a delivery of cortical hormone from the transplants. — Injection of an adrenal cortical extract of the SWINGLE and PFIFFNER-type (Ecortan) causes a similar hypercholesterolemia, and we must therefore be justified in supposing that both cases are due to the effect of the same factor (hormone)

in the adrenal cortex; this factor does not seem to be identical with desoxycorticosteronacetate.

The presence of a serum cholesterol increasing factor in adrenal cortex has not been previously established, although a few authors (MARANON and COLLAZO 1935, BERNHARDT and SIMPSON 1932) in brief experiments state that they have found a similar effect of a SWINGLE-PFIFFNER-preparation. In this connection it ought to be mentioned that in Morbus Cushing a hypercholesterolemia has been described together with adrenal hyperplasia (RUTISHAUER 1933, MENZEL 1934, RAAB 1938).

It has not been possible to solve the problem of the influence of the adrenal cortex function on serum cholesterol by means of the adrenalectomy experiments undertaken, but the experiments have shown that it cannot with certainty be excluded that the hypercholesterolemia demonstrated by earlier authors is an unspecific symptom.

Summary.

The cholesterol content has been determined in the serum of rabbits after transplantation of adrenals, injection of adrenal cortical preparations and removal of one or both adrenals. In special experiments it could be demonstrated that adrenal cortical hormone was really transferred by the transplantation technique employed. After transplantation of adrenals and after injection of adrenal cortical extract of the SWINGLE-PFIFFNER-type a hypercholesterolemia could be demonstrated, as an expression of the presence of a hormone with cholesterol-increasing effect in the adrenal cortex; this hormone is not identical with desoxycorticosteronacetate. After adrenalectomy no certain alterations in the serum cholesterol, corresponding to decreased or excluded adrenal cortical function, could be demonstrated.

References.

BAUER, J. and G. D. A. BUTTU, Z. klin. Med. 1932. 122. 601.
BAUMANN, E. J. and O. M. HOLLY, J. Biol. Chem. 1923. 55. 457.
BERNHARDT, H. and S. L. SIMPSON, Klin. Wschr. 1932. 2069.
CHAUFFARD, A., G. LAROCHE and A. GRIGAUT, C. R. Soc. Biol. Paris 1914. 76. 529.
HARROP, G. A., J. J. PFIFFNER, A. WEINSTEIN and W. W. SWINGLE, Science 1931. 73. 683.
HESSE, H. and M. TRIER, Ugeskr. Læg. 1937. 99. 935.

HOFFMEYER, J., *Acta Physiol. Scand.* 1944. 7. 313.
HUECK, W., *Verh. d. d. path. Ges.* 1914. 17. Tag., 149.
LANDAU, M. and J. W. MCNEE, *Beitr. path. Anat.* 1914. 58. 667.
MARANON, G. and S. A. COLLAZO, *Rev. franç. Endocrin.* 1935. 13. 1.
MEDVEI, C. V., *Z. klin. Med.* 1935. 128. 58.
MENZEL, W., *Ibidem.* 1937. 131. 565.
RAAB, W., *Klin. Wschr.* 1939. 18. 611.
Randles, F. S. and A. KNUDSON, *J. Biol. Chem.* 1928. 78. 89.
ROTHSCHILD, M. A., *Beitr. path. Anat.* 1914. 58. 667.
RUTISHAUER, E., *Dtsch. Arch. klin. Med.* 1933. 175. 640.
SILBERSTEIN, F., M. WACHSTEIN and F. GOTTDENKER, *Z. ges. exp. Med.* 1932. 81. 133.
SWINGLE, W. W. and J. J. PFEIFFNER, *Science* 1930. 71. 321.
THADDEA, S. and W. FASSHAUER, *Arch. exp. Path. Pharmak.* 1936. 182. 477.

From the Department of Medical Chemistry,
University of Helsingfors.

Contribution to Thunberg's Method of Estimating Citric Acid.

On the "Systematic Deviation" of the Ci-curve and
the Estimation of the Ci-value from the Curve.

By

LARS HAGELSTAM.

Received 20 April 1945.

The estimation of very small quantities of citric-acid (in the following abbreviated "Ci") in biological fluids was not possible till THUNBERG (1929) had published his enzymo-chemical method of estimating Ci. The method is assumed to be known, and no detailed description will be given here, only a few introductory words and the terminology.

The phosphate extract of cucumber-seed, the "enzyme solution", does not only contain Ci dehydrogenases, but also other dehydrogenases and their substrates, the "spontaneous donors". In the reaction process a distinction is therefore drawn between "Ci-activity" and "spontaneous activity". The shape of the Ci-curve is determined by a concurrence of these two factors. The curve consists of a horizontal part, the "level line", and an "ascending leg". The point where the ascending leg leaves the level line is called the "contact point". The "X-curve", i. e. the curve for the "X-solution" in which the Ci-content is unknown, is compared to the "standard curve", i. e. the curve for the "standard solution", in which the Ci-content is known. THUNBERG's original and still most commonly used method of estimating the Ci-value is based on the supposition that equal quantities of Ci correspond to equally long decoloration-times in the X- and standard curves. If this supposition were literally valid, the X- and standard curves

would be congruous when the Ci-content is equally great in the X- and standard solutions. But, working with biological fluids one finds that the curves, as a rule, are incongruous even in those cases, where the Ci-content, calculated as a mean value of several test-tubes of the X-curve, is the same in the X- as in the standard solutions.

The said incongruity lies in the fact that the level line lies on a higher plane in the X- than in the standard curve, and that the tubes with a smaller quantity of X-solution are decoloured relatively quicker than the corresponding tubes in the standard series. This implies that the X curve slopes more steeply towards

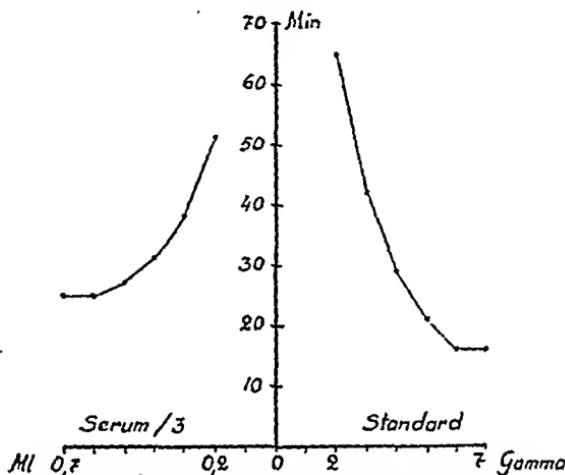


Fig. 1. Estimation: The yield of the tube with 0.2 ml is calculated for undiluted serum 39.00 γ/ml of Ci, 0.3—32.00, 0.4—29.25, 0.5—25.20, 0.6—22.50 γ/ml .

the ordinate and that one, when estimating the Ci-value for each separate tube in the X-curve, gets a relatively smaller yield from a bigger quantity of X-solution, and a relatively bigger yield, the smaller quantity of X-solution a tube contains. The phenomenon is called the "systematic deviation of the X-curve", in German "Gang" [SJÖSTRÖM (1937)]. This incongruity of the curves does not assert itself so clearly when the object of the examination is urine [ÖSTBERG (1931)]. In blood serum tests the phenomenon appears regularly, and has often a disturbing effect by introducing a certain insecurity in the estimation, fig. 1.

It seemed natural to assume that the systematic deviation was due to the fact that the examined fluid (urine, blood serum) contains substances that retard or accelerate the reaction. The retarding or accelerating influence on the reaction in solutions with known Ci-content of a great number of mineral salts and

organic matters has been studied by THUNBERG (1929), ÖSTBERG (1931, 1934), SCHERSTÉN (1932), NORDBÖ and SCHERSTÉN (1932), LENNÉR (1934) and GRÖNVALL (1937). It became evident that the substances which affected the reaction were not found in blood serum in such quantities, that they could considerably influence the process. It has however been generally accepted that the systematic deviation must be due to the action of retarding or accelerating substances in the biological fluid to be examined. THUNBERG (1933) assumed the existence of retarding substances. ÖSTBERG (1934) pointed out that the influence of these substances on the curve a priori makes it most unlikely that a value in the middle of the curve would be the correct one. Having closely studied the character of the Ci-curve, LENNÉR (1934) observed that it is easy to understand that the laws of the course of the curve are difficult to find, as all the factors which influence the course of the curve vary in an uncontrollable manner and influence each other mutually. MÅRTENSSON (1940) has also been working on this problem, and he thought the main cause of the systematic deviation to be that the serum, except Ci, contains other substances serving the dehydrogenases of the enzyme solution as donors; these small quantities of other donors would be more effective higher up on the curve, where the Ci-quantity is smaller. He also assumed a retardation in the lower part of the curve. The hydrogen-ion concentration of the examined fluid has also been proved to affect the process, ÖSTBERG (1931), ANDERSSON (1938). MÅRTENSSON (1940) however arrived at the conclusion that the existent variations in pH in blood serum do not affect the decoloration-time.

Among the factors which exclusively affect the reaction in the X-curve without being active in the standard solution, the influence of the colloid-content in the X-solution has not earlier been systematically examined, neither has the effect the serum colloids can be thought to have on the process been clearly stressed. It is, however, evident that the presence of 0.2—0.6 ml serum, respectively serum dilution, in a total fluid amount of 2.0 ml per tube in the X-series, through its colloid-content must affect the reaction differently than the aqueous solution of crystalline Ci, used in the same quantities in the standard solution. The colloidal enzyme solution is added to all tubes in equal quantity, and cannot affect the reaction differently in the X-curve than in the standard curve.

In an earlier work [HAGELSTAM (1944)], the author has shown that a 6 % solution of albumen ovi pulverisatum causes a systematic deviation which completely corresponds to that caused by serum. The observation led to the following attempt to find a general rule for the influence of the colloid content on the systematic deviation.

Methodics.

The Ci-determination has been made according to the method currently used in Lund.

The enzyme: Seed of the cucumber "Muromska", Swedish state control I class, power of germination 99 %, from OY Björklund AB, Helsingfors, was used. The seeds were manually shelled and placed in open glass for 24 hours. They were then manually pounded in a mortar; the degree of crushing was kept as constant as possible throughout the whole of the test series. For the extraction 0.57 % K₂HPO₄ was used, in proportion 1 gr shelled cucumber-seed per 7 ml of phosphate solution. The mixture was kept over night in refrigerator and was, when used, 16—22 hours old. After 20 min. centrifuging the clearer middle layer was separated and cooled in a glass in ice-water in a dark cupboard for 30 min., after which the enzyme was ready for use.

Methylene blue: The original solution 1 : 5,000 was made with distilled water, the diluted solution 1 : 50,000 was every day prepared in the same manner.

Diluted Ringer solution: 8.5 gr NaCl, 100 mgr CaCl₂, and 75 mgr KCl per 3,000 ml distilled water was used for preparing the X- and standard solutions.

The tests were carried out in constant electric light from 4 lamps attached to a stand 1.5 m from the water-bath. The temperature of the water-bath was kept constantly at +35° C. The atmospheric pressure in the tubes was 3—4 mm Hg after the evacuation. The tubes were alternately evacuated in the X- and standard series, beginning from the tubes with the smallest Ci-value.

By minute observation of these rules stability was obtained in the enzyme and generally in the reaction process, throughout the whole test series. The level line of the standard curve stayed at 14—15 min. and the spontaneous decoloration time at 80—100 min. The contact point lay at 0.6 ml in both the X- and standard curves.

A number of solutions of *Albumen ovi pulverisatum*, *Gummi arabicum* and *gall of swine* were used in studying the influence of the colloids on the reaction. By the THUNBERG method Ci could not be proved in a 3 % solution of alb. ovi pulv. or in a 2 % solution of gummi arab. By the pentabromacetone method, as modified by PUCHER, SHERMAN and VICKERY, it was established that gall of swine does not contain Ci, neither could the presence of Ci in 10 % solutions of alb. ovi pulv. or gummi arab. be detected by the same method.

Diluted Ringer solution was used in preparing the different colloidal solutions; to them was added 10 γ/ml of Ci. These colloidal solutions with known Ci-content were used as X-solutions and compared to the standard solution (10 γ/ml of Ci), when the Ci-yield of each tube in the X-series could be determined. In these experiments could further the amount of X-solution, which theoretically ought to have given the quantitatively right yield (10 γ/ml), be read directly off the curve; this amount corresponding to the point of the X-curve, where the X- and standard curves are on the same distance from the ordinate. At each experiment the Ci-yield was calculated for the tubes with 0.2, 0.3, 0.4, 0.5 and 0.6 ml of X-solution, tube by tube. Then the yield was calculated as a mean value of the following combinations of tubes: 1) 0.2, 0.3. 2) 0.2, 0.3, 0.4. 3) 0.2, 0.3, 0.4, 0.5. 4) 0.3, 0.4. 5) 0.3, 0.4, 0.5. 6) 0.3, 0.4, 0.5, 0.6. 7) 0.4, 0.5. 8) 0.4, 0.5, 0.6. 9) 0.5, 0.6, that is, of all existing combinations of 2, 3 or 4 tubes following each other in the series. The average of the obtained values was calculated in every series of tests. The average of the time values for the level lines were as well calculated in every test series, after which the figure for the expression $\frac{\text{the level of the X-curve}}{\text{the level of the standard curve}}$, in the following called *the level quotient*, was calculated from the above mentioned mean value of the time values.

Own Researches.

Albumen Ovi Pulverisatum, Gummi Arabicum and Gall of Swine as Causes of Systematic Deviation.

In the following report and in the tables 1—4 all figures on the results are means of the values found in the respective tests series.

Of *Albumen ovi pulverisatum* two different preparations were used, viz. "SCHERING-KAHLBAUM" and "Dr. THEODOR SCHUCHARDT, Görlitz", the former in concentration 6 : 100 and 3 : 100, the latter in concentration 6 : 100 (v. table 1).

The preparation *Albumen ovi pulverisatum* "SCHERING-KAHLBAUM" in concentration 3 : 100 proved itself in 10 tests to be the cause of a considerable systematic deviation. The yield of the tube with 0.3 ml of Ci-albumen solution was 13.43 γ/ml of Ci, instead of 10 γ/ml, that is 34.3 % too big. In the tube with 0.4 ml the yield was 18.8 % and in the tube with 0.5 ml 5.8 % too big, while the yield of the tube with 0.6 ml was 5 % too small. On an average the level line of the X-curve was at 17.7 min., that of the standard curve was at 15.4 min., which implies the level quotient 1.15. The right yield 10 γ/ml would theoretically have been obtained from a tube with 0.55 ml of Ci-albumen solution. Of all the different mean values of the yield of 2, 3 or 4 consecutive

Table 1.
Experiments on Albumen ovi pulverisatum.

Colloidal solution	Number of tests	Mean value of the yield of the different tubes										The yield calculated as a mean value for several tubes					Correct yield at ml	Mean value of level line	St.	X	
		0.2	0.3	0.4	0.5	0.6	0.2, 0.3	0.2	0.2, 0.3	0.3	0.3, 0.4	0.3	0.3, 0.4	0.4, 0.5	0.5, 0.6	0.4					
Albumen ovi pulverisat. "Scherling," Kahlbaum, 3 : 100 + 10 γ /ml of Ci	10	—	13.13	11.88	10.58	9.50	—	—	—	—	—	12.06	11.96	11.35	11.23	10.65	10.04	0.55	15.4	17.7	1.15
Albumen ovi pulverisat. "Scherling," Kahlbaum, 6 : 100 + 10 γ /ml of Ci	5	—	14.33	12.03	10.44	9.17	—	—	—	—	—	13.19	12.27	11.50	11.25	10.55	9.81	0.53	15.2	18.4	1.21
Albumen ovi pulverisat. "Dr. Theodor Schuchhardt," Görlitz" 6 : 100 + 10 γ /ml of Ci	5	11.00	10.86	10.36	9.64	8.67	10.94	10.72	10.45	10.58	10.27	9.87	9.97	9.54	9.16	0.43	14.6	18.2	1.25		

tubes the average for the tubes with 0.5 and 0.6 ml proved themselves to give the best value, 10.04 γ/ml , the result being only 0.4 % too big.

5 tests were made with the same preparation in concentration 6 : 100. The yield of the tube with 0.3 ml of Ci-albumen solution was now 43.3 % too big, and that of the tube with 0.6 ml 8.3 % too small. This stronger concentration showed a higher level line, the level quotient being 1.21. The theoretically correct yield ought to have been found in a tube with 0.53 ml, and the best mean value was actually calculated from the tubes with 0.5 and 0.6 ml, it being 9.81 γ/ml or 1.9 % too small.

We find consequently that the more concentrated albumen solution, in spite of having the same Ci-content, shows a higher level line and a greater systematic deviation. By increased albumen content the X-curve is turned in towards the ordinate, and the curves of these albumen solutions with different concentrations intersect at a point where the right Ci-yield 10 γ/ml is to be found, that is at 0.53—0.55 ml of Ci-albumen solution. For further particulars see the tests with gummi arab.

The preparation Alb. ovi pulv. "Dr. THEODOR SCHUCHARDT, Görlitz" in concentration 6 : 100 proved itself in 5 tests to have, to a certain extent, different properties than the same concentration of the "SCHERING-KAHLEBAUM" preparation. A systematic deviation was clearly evident, but there was an obvious difference in degree. From the tube with 0.3 ml was now obtained a yield that was only 8.6 % too big, while that from the tube with 0.6 ml was 13.3 % too small. The point of the right value had in conformity to this moved higher up on the curve, and lay at the theoretical amount of 0.43 ml. The level quotient was 1.25. The best yield was calculated from the tubes with 0.4 and 0.5 ml and was only 0.3 % too small.

As no analysis was made of the two albumen preparations as to their content of water, mineral salt etc., it would be difficult to point out the cause of this difference in their influence on the reaction. But, taking the following into account, it is not unwarranted to assume that the difference in influence on the process was due to quantitative or qualitative differences in the colloids, depending on differences in the production.

With *gummi arab.* 60 tests in all were made, 10 tests with each of the concentrations 0.13 : 100, 0.25 : 100, 0.50 : 100, 1 : 100, 2 : 100 and 3 : 100 (v. table 2).

Table 2.
Experiments on *Gummi arabicum*.

Colloidal solution	Number of tests	Mean value of the yield of the different tubes						The yield calculated as a mean value for several tubes						Mean value of level line	Mean value of level line				
		0.2	0.3	0.4	0.5	0.6	0.2, 0.3	0.2	0.3	0.4	0.3	0.4	0.5	0.4, 0.5	0.4	0.5	0.6	St.	X
Gummi arabicum 0.13 : 100 + 10 γ/ml of Ci	10	11.13	10.83	9.88	9.22	8.23	11.05	10.63	10.24	10.36	9.98	9.54	9.55	9.10	8.72	0.38	14.5	20.6	1.38
Gummi arabicum 0.25 : 100 + 10 γ/ml of Ci	10	12.07	11.03	9.88	8.88	7.97	11.10	10.84	10.31	10.46	9.93	9.44	9.38	8.91	8.43	0.38	14.4	21.7	1.51
Gummi arabicum 0.50 : 100 + 10 γ/ml of Ci	10	12.33	11.23	9.76	8.48	7.47	11.59	10.89	10.25	10.51	9.83	9.24	9.22	8.57	7.98	0.38	14.6	24.8	1.70
Gummi arabicum 1.09 : 100 + 10 γ/ml of Ci	10	12.42	10.27	9.45	8.20	7.25	11.41	10.65	9.96	9.86	9.31	8.79	8.83	8.30	7.73	0.31	14.7	25.4	1.73
Gummi arabicum 2.00 : 100 + 10 γ/ml of Ci	10	13.17	10.60	9.18	7.78	6.63	11.89	10.91	10.09	9.89	9.12	8.55	8.49	7.86	7.21	0.34	14.7	29.7	2.02
Gummi arabicum 3.00 : 100 + 10 γ/ml of Ci	10	13.10	10.37	8.75	7.30	6.24	11.74	10.74	9.87	9.56	8.81	8.16	8.03	7.43	6.77	0.32	14.3	32.9	2.33

While the yield of the tube with 0.2 ml at the concentration 0.13 : 100 was 14.3 % too big, it was 31 % too big at the concentration 3 : 100. At the same concentrations the yield of the tubes with 0.6 ml was 17.7 %, respectively 37.6 % too small. By rising colloid concentration the yield of the tube with 0.2 ml increased. The yield of the tube with 0.3 ml showed no clear tendency to increase or decrease, while the yield of the tubes with 0.4, 0.5 and 0.6 ml diminished by a rising colloid content.

From the table can be seen that a rising concentration of gummi arab. causes an increase of the systematic deviation. A turning of the curve towards the ordinate takes place at the

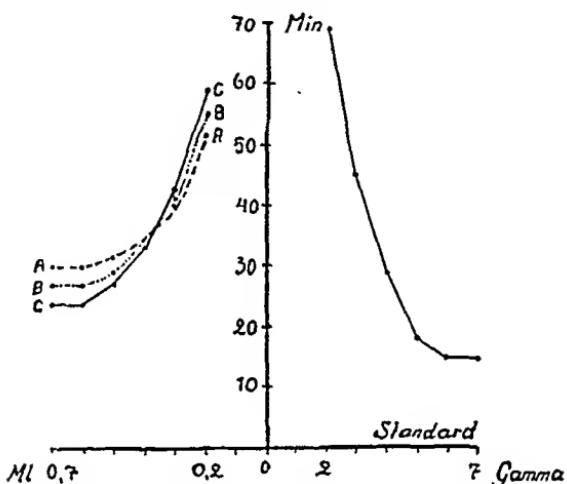


Fig. 2. 10 γ/ml of Ci in Gummi arab.: A 2 %, B 1 %, C 0.5 %.

same time as the level line rises, the turning happens about a point within the range of the theoretically right yield, which at the concentration 3 : 100 was at 0.32 ml. This is illustrated by fig. 2.

The best mean value from several consecutive tubes was obtained at the concentrations 0.13 : 100, 0.25 : 100 and 0.50 : 100 from the combination 0.3, 0.4 and 0.5 ml. At the concentrations 1 : 100, 2 : 100 and 3 : 100 the best mean value was obtained from the combination 0.2, 0.3, 0.4 and 0.5 ml. Calculated in this manner the yield was remarkably exact, we find the biggest error at the concentration 0.5 : 100, where it was 1.7 %. At the concentration 0.13 : 100 the error was only 0.2 %. We find that the level quotient in those cases, where the best yield was obtained from the combination 0.3, 0.4 and 0.5, was between 1.38 and 1.70, while it in those cases, where the best yield was obtained from the com-

bination 0.2, 0.3, 0.4 and 0.5, was between 1.73 and 2.33. The exact yield was theoretically obtained, at the concentration 0.13 : 100 and the level quotient 1.38, of 0.38 ml, while the corresponding point at the concentration 3 : 100 and the level quotient 2.33 lay at 0.32 ml. It has consequently been shown also in the tests with gummi arab. that an upward displacement of the level line involves a displacement of the point for the exact yield in the same direction on the curve.

Gall of swine was used in the concentrations 1 : 3,000, 1 : 2,000, 1 : 1,000, 1 : 500 and 1 : 100 (v. table 3). With each of these solutions 5 tests were made. In concentration 1 : 3,000 gall of swine gave a fairly insignificant deviation of the curve, the yield of the tube with 0.2 ml being only 8 % too big, while the yield of 0.6 ml was 2.8 % too small. The level quotient was on this occasion as small as 1.06 and the point for the exact yield lay as low down on the curve as at 0.55 ml. At the concentration 1 : 100 the highest and lowest points on the curve showed, as a consequence of the greater systematic deviation, considerable errors in the calculated yield. From the tube with 0.2 ml was now obtained a yield which was 25 % too big, from the tube with 0.6 ml, on the other hand, a value which was 34.6 % too small. The level quotient was now at 1.94 and the point for the exact yield lay as high up on the curve as at 0.34 ml. Consequently the results tend in the same direction as the tests on albumen and gummi arab. In all tests we find a distinct systematic deviation which increases with the colloid concentration, at the same time as the level line rises and the point for the exact yield is displaced upwards on the curve.

Conclusions:

1. Colloidal solutions cause a systematic deviation of the Ci-curve.
2. Different colloids in the same concentration cause deviations of unequal strength.
3. If the concentration of a given colloid is increased, the level line and the point for the theoretically correct yield are displaced upwards on the curve, at the same time as the systematic deviation increases.
4. The systematic deviation of the Ci-curve is due to an acceleration of the reaction process in the upper part of the curve and a retardation in its lower part. When the systematic deviation increases, the curve turns about a point that lies between 0.30 and 0.55 ml, if the contact point lies at 0.6 ml.

Table 3.
Experiments on gall of swine.

Colloidal solution	Number of tests	Mean value of the yield of the different tubes	The yield calculated as a mean value for several tubes										Mean value of level line	St.	X	
			0.2	0.3	0.4	0.5	0.6	0.2, 0.3	0.2, 0.3, 0.4	0.2, 0.3, 0.4, 0.5	0.3, 0.4	0.3, 0.4, 0.5	0.3, 0.4, 0.5, 0.6			
Gall of swine 1:3,000 + + 10 γ/ml of Ci	5	10.80	10.53	10.56	10.44	9.72	10.87	10.63	10.59	10.55	10.51	10.31	10.50	10.24	10.08	0.55
Gall of swine 1:2,000 + + 10 γ/ml of Ci	5	12.00	11.07	10.42	9.58	8.51	11.54	11.17	10.77	10.75	10.39	9.90	10.00	9.50	9.05	0.45
Gall of swine 1:1,000 + + 10 γ/ml of Ci	5	12.40	11.00	10.10	9.00	7.87	11.70	11.17	10.63	10.55	10.03	9.49	9.55	8.99	8.44	0.41
Gall of swine 1:500 + 10 γ/ml of Ci	5	13.65	11.47	9.75	8.24	7.07	12.56	11.62	10.78	10.61	9.82	9.13	9.00	8.35	7.66	0.38
Gall of swine 1:100 + 10 γ/ml of Ci	6	12.50	10.93	9.20	7.66	6.54	11.72	10.88	10.08	10.07	9.26	8.63	8.43	7.84	7.14	0.34

The Level Quotient and the Ci-yield.

If the material now is arranged according to the magnitude of the numeral value of the level quotient an interesting fact will appear (v. table 4). A certain colloid in weak concentration may have as great an influence on the height of the level line and the level quotient as a more than 100 times more concentrated solution of another colloid. Alb. ovi pulv. "SCHERING-KAHLBAUM" in concentration 6 : 100 gives e. g. a lower level quotient than gall of swine in concentration 1 : 2,000; the difference in the content of dry substance of the solutions is in this case much greater even than what is shown by the numeral value of the concentrations.

As the point for the exactly right yield by a rising level quotient is displaced upwards on the curve, it is to be expected that the best mean value for the yield from consecutive tubes will be obtained from tubes with smaller amount of X-solution, the higher the level line of the X-curve lies. This is also the case. In the table the combinations of tubes whose mean value came nearest to the correct value ($10 \gamma/ml$) have been marked by horizontal lines. We find that by a rising level quotient also this best combination is successively displaced upwards on the curve. If the level quotient is small, 1.06—1.21, the mean value for the tubes with 0.5 and 0.6 ml gives the best yield. By a level quotient of 1.24—1.25 the best mean value is obtained from the tubes with 0.4 and 0.5 ml. If the level quotient is between 1.38 and 1.70, the mean value for the tubes with 0.3, 0.4 and 0.5 ml is the best. If the level quotient rises to 1.73—2.33, the mean value must be calculated from the tubes with 0.2, 0.3, 0.4 and 0.5 ml, to be as exact as possible. It is remarkable that, in spite of a considerable systematic deviation the yield, by observation of this rule, comes so near the correct one in all of the 14 test series. On an average the error was 0.81 %, and in no case it was bigger than 1.9 %.

Conclusion: When working on colloidal solutions, the numeral value of the level quotient determines which tubes ought to be used for calculation of the Ci-value. By a rising level quotient the best combination of tubes is regularly displaced upwards on the curve.

Table 4.
Estimation of the Ci-value with consideration of the level quotient.

Level quotient	The best yield is calculated from the tubes with					The best yield of 10 γ/ml Ci	Percentage of error	Theoretically right yield at ml	Colloidal solution
	0.2	0.3	0.4	0.5	0.6				
1.06						10.08	0.8	0.55	Gall of swine 1 : 3,000 + 10 γ/ml of Ci
1.15						10.04	0.4	0.55	Albumen ovi pulv. "Scherling Kahlbaum" 3 : 100 + 10 γ/ml of Ci
1.21						9.81	1.9	0.53	Albumen ovi pulv. "Scherling Kahlbaum" 6 : 100 + 10 γ/ml of Ci
1.24						10.00	0	0.45	Gall of swine 1 : 2,000 + 10 γ/ml of Ci
1.25						9.97	0.3	0.43	Albumen ovi pulv. "Dr. Theodor Schuchardt, Görlitz" 6 : 100 + 10 γ/ml of Ci
1.38						9.98	0.2	0.38	Gummi arabicum 0.13 : 100 + 10 γ/ml of Ci
1.42						10.03	0.3	0.41	Gall of swine 1 : 1,000 + 10 γ/ml of Ci
1.51						9.93	0.7	0.38	Gummi arabicum 0.25 : 100 + 10 γ/ml of Ci
1.67						9.82	1.8	0.38	Gall of swine 1 : 500 + 10 γ/ml of Ci
1.70						9.83	1.7	0.38	Gummi arabicum 0.5 : 100 + 10 γ/ml of Ci
1.73						9.96	0.4	0.34	Gummi arabicum 1 : 100 + 10 γ/ml of Ci
1.94						10.07	0.7	0.34	Gall of swine 1 : 100 + 10 γ/ml of Ci
						(10.08)	(0.8)		
2.02						10.09	0.9	0.34	Gummi arabicum 2 : 100 + 10 γ/ml of Ci
2.33						9.87	1.3	0.32	Gummi arabicum 3 : 100 + 10 γ/ml of Ci

Is the Systematic Deviation Influenced by Changes in the Colloidal Properties of a Given Colloid?

Having found that the nature of the colloid that causes the systematic deviation is of the greatest importance as to the degree of the deviation, the height of the level line and the level quotient, it may be of interest to investigate whether changes in the composition of a given colloidal solution affect the above mentioned phenomena in the curve. As material for these tests vital albumen of hen's egg was chosen. THUNBERG (1941) has proved that the albumen of hen's egg contains about $10 \gamma/ml$ of Ci. The albumen was therefore used in undiluted form. Changes in the colloidal composition of the albumen of hen's egg were brought about in two ways:

1. by heating to $63.0-63.5^\circ$ for 30 min., whereby a distinct opalescence could be observed,
2. by freezing in liquid air.

The opalescence observed after the heating implies a change in the colloidal composition of the albumen. The freezing of a colloidal solution causes a change from a sol- to a gel-state, as has been pointed out by SCHADE (1923). That these manipulations of the material to be tested really affected the degree of the systematic deviation is shown in table 5. In both cases the degree of the deviation was augmented and the lowest point of the curve was displaced upwards.

Conclusion: The systematic deviation is affected as to its degree by changes in the colloidal properties of the tested material.

Is the above Stated Rule for Estimating the Ci-value of Colloidal Solutions Valid also for Serum?

When estimating the Ci-content of blood serum by the THUNBERG method, one must ask oneself, whether the value obtained really expresses the pure Ci-content. Two factors ought then to be taken into consideration. It is likely that, together with Ci, also the iso-citric acid and the cis-aconitic acid are determined by the method, BREUSCH (1937), quoted MÅRTENSSON (1940). Investigations into the ratio of these acids to Ci in tissues has shown that 75—90 % consist of Ci, BREUSCH (1937), MARTIUS (1938), JOHNSON (1939), quot. MÅRTENSSON (1940). In 13 estimations of Ci in serum from arterial blood MÅRTENSSON (1940)

Table 5.
Increase of the systematic deviation after freezing or heating the albumen.

Test N:r	Colloidal solution	Ci-value calculated from tubes with ml					Height of level line in min.	
		0.2	0.3	. 0.4	0.5	0.6	St	X
22.	Albumen ovi vitalis	11.33	9.75	8.40	7.50	14	25	
	" heated for 30 min. to 63.5°	11.33	9.25	7.80	6.83		29	
56	Albumen ovi vitalis	15.50	12.33	9.75	8.20	7.90	16	28
	" heated for 30 min. to 63.5°	16.00	12.00	9.50	8.00	6.83		29
57	Albumen ovi vitalis	14.50	12.00	9.75	8.00	6.83	14	27
	" heated for 30 min. to 63°	15.00	11.67	9.25	7.80	6.50		29
58	Albumen ovi vitalis	16.50	13.00	10.25	8.60	7.33	15	25
	" heated for 30 min. to 63°	17.00	12.66	9.75	8.00	6.66		29
69	Albumen ovi vitalis	14.50	12.33	10.00	8.40	7.17	14	25
	" freezed in liquid air	15.50	12.00	9.75	8.20	7.00		26
70	Albumen ovi vitalis	14.50	13.00	10.59	8.80	7.50	15	24
	" freezed in liquid air	15.50	13.00	10.25	8.20	7.33		26

found that the pentabromeacetone method, as modified by PUCHER, SHERMAN and VICKERY (in the following abbreviated PSV), gave, on an average, a 16.5 % smaller Ci-yield than THUNBERG's enzymic method. In 6 parallel estimations with venous blood, JOSEPHSON (1941) found with the PSV method on an average $25.5 \gamma/ml$, with the THUNBERG method on an average $23.9 \gamma/ml$ (the average is calculated by the author from JOSEPHSON's figures). No other comparative researches on serum are known to me.

Secondly, Ci used in the standard solution is weighed together with its crystal water, which is 8.576 % of the weighed Ci-amount. The figure obtained as an expression for the Ci-content in serum ought consequently to be too big, estimated as water free Ci the right value would be 91.424 % of that read off the curve. But THUNBERG (1936) has pointed out the possibility of the natural form of Citric acid being an ortho-acid and of the water, which is usually considered as crystal water, being intra-molecularly bound. If this were the case, the estimated Ci-value would be correct, with exception of an error caused by eventual presence of iso-citric acid and cis-aconitic acid. In the chemical PSV method we have a possibility of determining the Ci-content in blood serum, without the result being influenced by the above mentioned factors.

In order to get a conception of whether the THUNBERG method actually gives as exact a result when working with blood serum as with artificial colloidal solutions, 14 parallel estimations of Ci in blood serum were made with the THUNBERG and PSV methods. To avoid eventual errors caused by unsuitable dilution of the serum tests, the Ci-content of the serum tests was first determined according to PSV, after which the dilutions for the THUNBERG tests were made so that the X-solution contained as exactly as possible $10 \gamma/ml$ of Ci. The result is shown by table 6.

Although the Ci-content in the serum dilutions is almost exactly $10 \gamma/ml$, the X-curves often show rather a high level line in relation to the standard curves, the level quotient often rising above 2. The expression used by SJÖSTRÖM (1937), "sera with high level line", is consequently justified. The Ci-yield is indicated in the table as calculated for undiluted serum. We find that the systematic deviation is great in all the examined tests. This does not prevent that the Ci-content according to THUNBERG and according to PSV shows an astonishing agreement.

Table 6.
Comparison between Ci-estimations on serum tests according to Thunberg and according to Pucher, Sherman and Vickery.

Test	Dilution	Level quotient	Ci-yield of the different tubes				Average Ci for the tubes with 0.3 0.4 a. 0.5 ml	Average Ci with consideration of level quotient	Ci-value in serum acc. to P. S. V.	Remark
			0.2	0.3	0.4	0.5				
1	1:2	1.36	26.00	22.00	20.00	18.40	15.67	20.13	19.25	Aortitis lueticæ. Anaemia.
2	1:1.5	1.43	20.25	17.51	15.00	13.20	11.75	15.24	15.30	Infarct. cordis.
3	1:1.5	1.63	19.50	17.00	14.63	12.00	10.76	14.74	14.50	Aortitis lueticæ.
4	1:1	1.91	11.75	9.33	8.25	7.20	6.17	8.26	9.13	Lues.
5	1:1.5	1.44	18.00	16.50	14.03	12.60	10.76	14.58	14.58	Tub. renum amb.
6	1:2	2.40	26.00	20.00	16.00	13.20	11.34	16.40	18.80	Rheumatismus ac.
7	1:1.5	2.60	16.50	13.50	11.25	9.30	7.70	11.35	13.75	Tub. pulm.
8	1:2	2.14	26.00	20.67	17.50	14.40	12.34	17.52	19.64	Lues.
9	1:2	2.07	23.00	19.34	17.00	14.80	12.66	17.05	18.54	Bronchopneumonia.
10	1:2	1.04	29.00	24.67	20.50	17.60	—	20.30	20.90	Myciromatosis. Anaemia.
11	1:3	1.29	34.50	33.00	30.00	28.20	24.99	30.40	29.10	Healthy individual.
12	1:4	1.43	48.00	46.67	41.00	36.80	31.30	41.50	41.50	Healthy? individual.
13	1:2.5	1.79	30.00	27.50	23.13	19.50	16.67	23.38	25.03	Healthy individual.
14	1:2.5	1.29	32.50	29.18	25.63	23.00	20.43	25.94	24.32	Healthy individual.

If all the 14 tests are taken into account, the mean value for the Ci-content according to THUNBERG, calculated from the tubes with 0.3, 0.4 and 0.5 ml, is 0.429γ , or 2.119 % smaller than the mean value according to PSV, while the value according to THUNBERG, if calculated with consideration of the level quotient, is 0.143γ , or 0.706 % bigger than what it is according to PSV. If only the eight tests are taken into account, in which we find a difference between the THUNBERG values calculated in these two different manners, the following result is arrived at. The mean value for the Ci-content according to THUNBERG, calculated from the tubes with 0.3, 0.4 and 0.5 ml is 0.850γ , or 4.301 % smaller than the mean value according to PSV, while the value according to THUNBERG, if the level quotient is considered, is 0.026γ , or 0.131 % bigger than what it is, if determined according to PSV. It has been pointed out that, in order to get the best value, one ought to estimate the Ci-content from the tubes nearest the contact point, ÖSTBERG (1934), MÄRTENSSON (1940). But it is fully evident that, in order to get the result as exact as possible, the level quotient is to be taken into consideration. In these 8 tests the difference between the Ci-value according to THUNBERG and according to PSV is nearly 30 times bigger, if the Ci-value is stereotypically calculated from the tubes with 0.3, 0.4 and 0.5 ml of serum solution, than if the level quotient is taken into account. In whichever way the Ci-value was calculated in the THUNBERG tests, the correspondence between the Ci-values according to THUNBERG and according to PSV was remarkably good. As in these parallel tests the serum dilution in the THUNBERG tests was made so that the X-solution actually contained very nearly $10 \gamma/ml$ of Ci, and considering the exact yield that was obtained from the artificial colloidal solutions, I am inclined to assume my results to be correct. Larger tests series must give the proof.

Everyone who has been working on Ci-estimations according to THUNBERG's method knows that the X-solution (the serum dilution) ought to contain $10 \gamma/ml$ of Ci, as exactly as possible, if the result is to be correct. If the serum test is not diluted enough, i. e. if the Ci-content of the X-solution is bigger than the optimum $10 \gamma/ml$, the yield will be too small, and the error can be of considerable magnitude. If the test is too much diluted a not so exact yield is also easily obtained. These errors caused by wrong dilution of the X-solution constitute finally the weakness of the THUNBERG method.

Conclusions:

1. When determining Ci in blood serum according to THUNBERG's method the level quotient ought to be taken into account, in order to obtain the most exact result possible. The same laws, which have been stated above as bearing upon the estimation of the Ci-yield of artificial colloidal solutions, are valid also in this case. As a general rule it can be said that the Ci-value is to be calculated from the tubes with 0.5 and 0.6 ml if the level quotient is between 1.00 and 1.20; from the tubes with 0.4 and 0.5 ml if the level quotient is between 1.20 and 1.30; from the tubes with 0.3, 0.4 and 0.5 ml if the level quotient is between 1.30 and 1.70; and from the tubes with 0.2, 0.3, 0.4 and 0.5 ml if the level quotient is between 1.70 and 2.40. If the level quotient is still greater, it is advisable to calculate the value from the tubes with 0.2, 0.3 and 0.4 ml, all the time supposing that the contact point lies at 0.6 ml.

2. As the systematic deviation of the serum curves follows the same laws as the systematic deviation caused by artificial colloidal solutions, one is justified to draw the conclusion that the systematic deviation is caused by the serum colloids.

3. A comparison between the Ci-content in serum from venous blood of man estimated according to THUNBERG and according to PSV showed a great conformity. The mean value of the Ci-values was, by estimation according to THUNBERG and with consideration of the level quotient, 0.7 % greater than the mean value of the Ci-values estimated according to PSV.

Summary.

Having demonstrated in an earlier work that an artificial colloidal solution causes a systematic deviation in the THUNBERG Ci-curve, the author poses the question: is the influence of the colloid content determined by a law, and does such a law concern also the Ci-estimation in blood serum? Solutions of alb. ovi pulv. gummi arab. and gall of swine to which was added 10 γ/ml of Ci caused a systematic deviation of the Ci-curve. The systematic deviation increased if the concentration of a given colloidal solution was increased. Different colloidal solutions in the same concentration caused systematic deviations of unequal degree. The figure for the expression $\frac{\text{the level line of the X-curve}}{\text{the level line of the standard curve}}$, called

the level quotient, determines which tubes ought to be considered when estimating the Ci-content of the X-solution. By a higher figure of the level quotient the point for the exact value is regularly displaced upwards on the curve. Simultaneously the for the estimation of the Ci-value best combination of several consecutive tubes is displaced upwards on the curve. The systematic deviation of the Ci-curve is due to an acceleration of the reaction process in the upper part of the curve and a simultaneous retardation of the process in its lower part. A change in the colloidal properties of a given colloidal solution affects the degree of the systematic deviation.

Parallel estimations of Ci in blood serum according to THUNBERG and according to PSV show that the same laws rule the systematic deviation in artificial colloidal solutions and in blood serum. This shows that the systematic deviation of the serum curve is due to the serum colloids.

Parallel Ci-estimations with the THUNBERG and PSV methods on venous blood of man gave almost identical results.

References.

ANDERSSON, B., Studien über die Co-Zymase als Aktivator bei enzymatischen Dehydrierungen. Stockholm 1938.

BREUSCH: quot. MÄRTENSSON.

GRÖNVALL, H., Acta ophthalm. Khb. 1937. Suppl.

HAGELSTAM, L., Acta chir. scand. 1944. 90. 37.

JOHNSON, quot. MÄRTENSSON.

JOSEPHSON, B., Sv. Läkartidn. 1941. 38. 1376.

LENNÉR, A., Acta obstetr. scand. 1934. 14. Suppl. 1.

MARTIUS, quot. MÄRTENSSON.

MÄRTENSSON, J., Acta physiol. scand. 1940. 1. Suppl. 2.

—, Nord. Med. 1940. 5. 253.

NORDBÖ, R. und B. SCHERSTÉN, Skand. Arch. Physiol. 1932. 63. 124.

SCHADE, H., Die physikalische Chemie in der inneren Medizin. Dresden und Leipzig 1923.

SCHERSTÉN, B., Skand. Arch. Physiol. 1932. 63. 97.

SJÖSTRÖM, P., Acta chir. scand. 1937. 79. Suppl. 49.

THUNBERG, T., Biochem. Z. 1929. 206. 109.

—, Kungl. fysiograf. Sällsk. i Lund Förh. 1933. 3. 149.

—, Abderhalden Hand. Biol. Arb. meth. 1936. 4: 2. 2017.

—, Skand. Arch. Physiol. 1936. 74. 1.

—, Kungl. fysiograf. Sällsk. i Lund Förh. 1941. 11. 126.

—, Sv. Läkartidn. 1941. 38. 1226.

ÖSTBERG, O., Skand. Arch. Physiol. 1931. 62. 81.

—, Ibidem. 1934. 67. 265.

From the Biochemical Institute, Aarhus University, Denmark.

On the Activation of Pancreatic Lipase by Calcium Chloride at Varying pH.

By

FRITZ SCHÖNHEYDER and KIRSTEN VOLQVARTZ.

Received 23 April 1945.

In spite of an extensive literature on lipases several elementary questions concerning these enzymes have not yet been satisfactorily answered. In previous works (1943, 1944, 1945) we have shown that among neutral and saturated triglycerides tributyrin is hydrolyzed with the greatest initial velocity by lipases. The affinity of pig pancreas lipase for lower triglycerides in homogeneous solution is extremely low. On the other hand the affinity of this enzyme for an insoluble triglyceride is very great, its reciprocal value being very small compared with the substrate surface measured. In the present paper the influence of calcium chloride on the activity of pancreas lipase is investigated.

PEKELHARING (1912) assumed that CaCl_2 accelerated the lipolysis by removing the fatty acids, which were supposed to inhibit the reaction, as insoluble calcium soaps. WILLSTÄTTER, WALDSCHMIDT-LEITZ and MEMMEN (1923) have denied the correctness of this view and on the basis of experiments with triolein they postulate that calcium soaps are the actual activators, the calcium soaps being able to form complex adsorbates. Further they maintain that the activating action can only be demonstrated at alkaline reaction. WILLSTÄTTER's view is quoted in most handbooks. Systematic investigations into the influence of calcium salts on the activity of pancreas lipase towards a series of saturated triglycerides (C_2 — C_{18}) are not reported. Experiments of this kind may have significance by illustrating some sides of the function

of calcium ions and will therefore be given in the following report. The activating influence of calcium chloride can be stated for all triglycerides investigated both in homogeneous and heterogeneous systems and not only in alkaline but also in acid medium. For the triglycerides with the highest molecular weight the activation on the acid side of optimum pH is most pronounced. The presence of calcium ions apparently does not shift the position of optimum pH for the same triglyceride. With increasing number of carbon atoms in the fatty acids a displacement of optimum pH in alkaline direction is seen. No basis for WILLSTÄTTER's theory as to the mechanism of the calcium activation has been found.

Experimental.

The activity of lipase is determined mainly as in our previous works, SCHÖNHEYDER and VOLQVARTZ (1944, 1945), the amount of acid liberated during the reaction being neutralized by adding drop-wise 0.1 n NaOH, keeping pH almost constant. pH was measured by means of a protected glass electrode. The reaction mixtures were shaken horizontally (250 double oscillations per min.) in a water thermostat at $40^{\circ}\text{ C} \pm 0.5^{\circ}\text{ C}$. The system employed consisted of triglyceride, 1 to 3 cc of buffer solution, ± 50 mg CaCl₂ (0.5 cc of 10 % CaCl₂ solution), enzyme extract and 0.9 % NaCl solution, the total volume being 30 cc.

Substrates. Triacetin, tripropionin, tributyrin, tricaproin, tricaprin, trilaurin, trimyristin, tristearin and triolein were used for the experiments. Regardless of solubility 0.283 millimol triglyceride were used for each experiment. Only in the case of triacetin 0.565 millimol were used. The systems with triacetin and tripropionin were homogeneous, the rest heterogeneous.

Buffers. Acetic acid — sodium acetate, Michaelis' veronal buffer, borate — NaOH and glycine — NaOH, all 0.1 n, were employed.

Calcium chloride. The amount chosen has been found to give optimal activation in experiments with tripropionin and pig pancreas lipase.

Enzyme. Pig pancreas lipase has been prepared according to the method of WILLSTÄTTER and WALDSCHMIDT-LEITZ (1923). In the case of triacetin and tristearin the undiluted preparation was used. With the other triglycerides the original extract was diluted with 0.9 % NaCl in suitable degree to get reaction rates below 35 drops per 10 min. An analysis of the glycerol extract showed that it did not contain measurable amounts of calcium.

The initial reaction velocity v was calculated graphically by plotting number of drops of 0.1 n NaOH (1 cc = 44 drops) against time in min. During the beginning of the reaction the curve was always found to be linear and v was computed as number of drops added during the first 10 minutes of reaction. In the most acid range the reaction curve in all cases without calcium showed a tendency to flattening within

a short time. This tendency did not appear in more alkaline range. In all cases, however, it is possible to get an estimate of the initial velocity with needed accuracy. All the initial velocities measured have been corrected for spontaneous hydrolysis which especially in the case of the lower triglycerides in alkaline range is considerable. Although the majority of the systems investigated are heterogeneous the individual experiments can be reproduced with an accuracy about 5 %. The amount of enzyme used is small in relation to the surface of the fatty particles in the heterogeneous systems, the initial velocity being approximately proportional to amount of enzyme at the same weight of triglyceride per 30 cc of reaction mixture.

Results.

Fig. 1 includes the experiments on the pH-activity relation for the triglycerides investigated with and without calcium chloride. The values represented by x correspond to experiments with CaCl_2 , 0: without CaCl_2 . The nine triglycerides investigated are: triacetin (I), tripropionin (II), tributyrin (III), tricaproin (IV), tricaprin (V), triolein (VI), trilaurin (VII), trimyristin (VIII) and tristearin (IX). Triolein is included because it is commonly used as substrate. For each triglyceride the same amount of the same enzyme preparation was used in all the experiments. Only one preparation of defatted and dried pancreas tissue was the source of the different enzyme preparations. The ordinates give initial velocity in per cent of the maximal velocity in the system containing CaCl_2 . The maximal velocity was calculated graphically. It ought to be mentioned that the curves have been reproduced in other experiments.

It appears from these experiments, that the pH optimum for the hydrolysis of triacetin is about 7 and the optimum for tristearin about 8.8, thus showing a definite displacement of the pH optimum with increasing number of carbon atoms in the fatty acids in the triglyceride. The curves with triacetin are incomplete owing to a very great spontaneous hydrolysis at $\text{pH} > 8.6$. For one and the same triglyceride the addition of calcium chloride does not change the pH optimum for the enzymatic hydrolysis. In no case an inhibiting action of calcium chloride in acid range could be stated. On the contrary in the case of triglycerides containing fatty acids with 10 or more carbon atoms the activation in the most acid range is more pronounced than in alkaline range. In the experiments with trilaurin, trimyristin and tristearin the range of activity in acid medium is widened about one pH unit, the pH

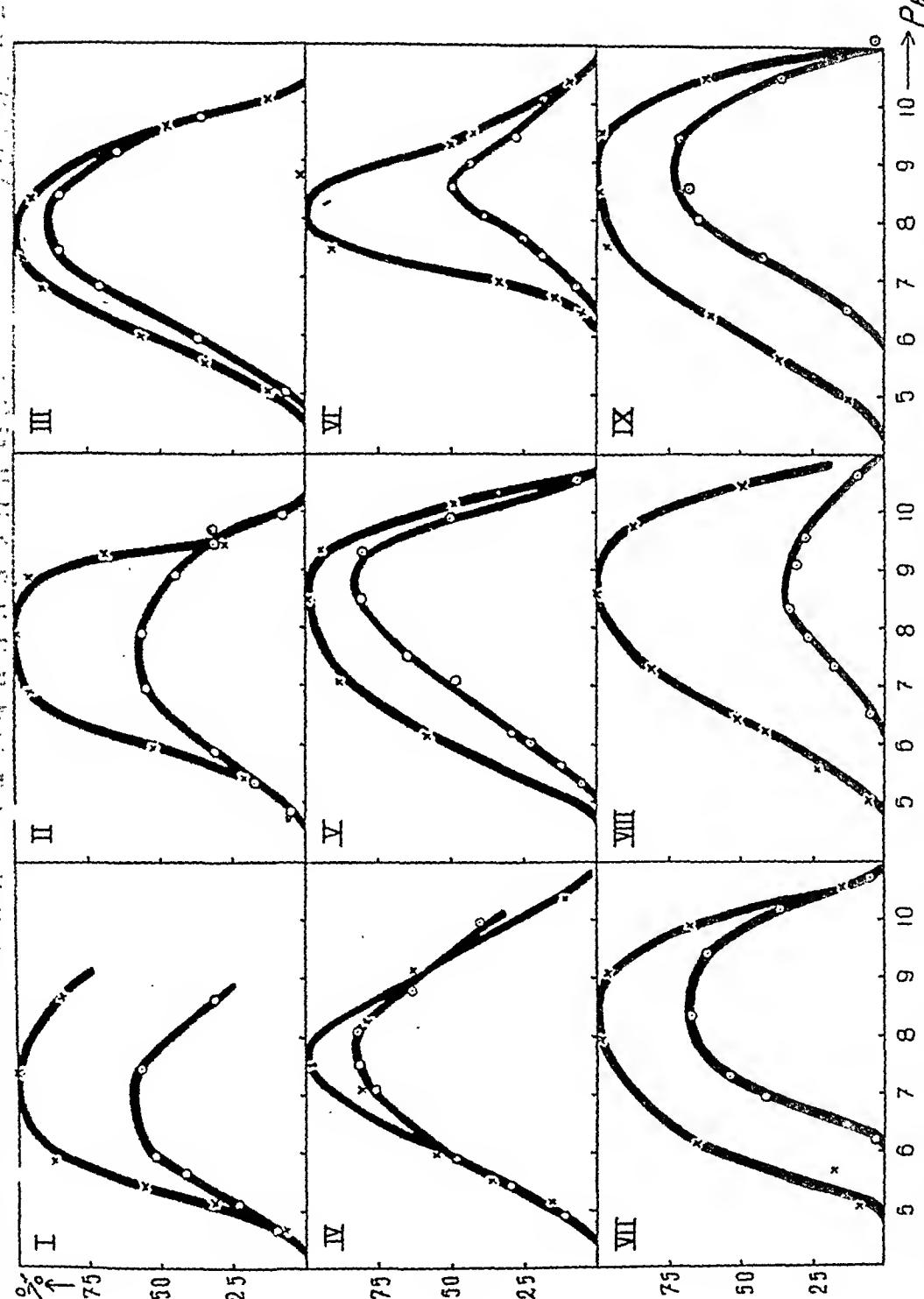


Fig. 1. Velocities of hydrolysis of triglycerides as functions of pH. o represents experiments without CaCl_2 ; x, addition of CaCl_2 .
 (I: triacetin, II: tripentan, III: tributyrin, IV: tricaprin, V: tricaproin, VI: triolein, VII: trimyristin, VIII: trimyristin, IX: tristearin).
 Abscissa: pH. Ordinate: Velocities in per cent of the maximal velocity in systems containing CaCl_2 .

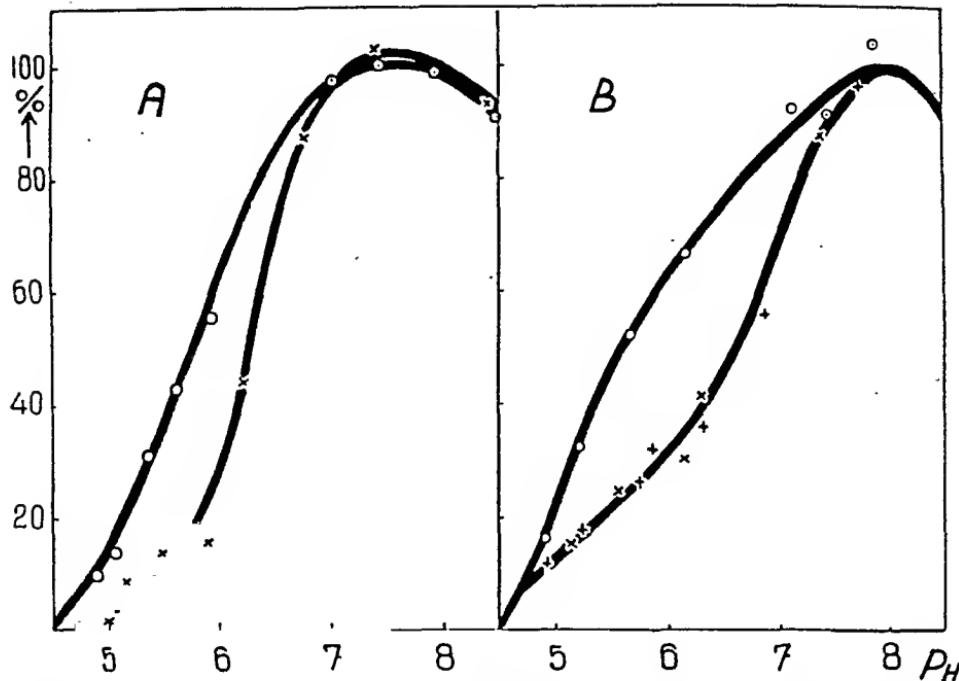


Fig. 2. Velocities of hydrolysis of triglycerides without and with addition of some higher fatty acids. A, tripropionin. o = tripropionin alone, x = addition of 0.1 millimol lauric acid. B, tricaproin. o = tricaproin alone, + = addition of 0.1 millimol lauric acid, x = addition of 0.1 millimol stearic acid. Abscissa: pH. Ordinate: Velocities in per cent of the maximal velocity in systems with no addition.

range of activity for these triglycerides after addition of CaCl_2 thus being of the same extension as in the case of the lower saturated triglycerides.

When no appreciable lipase activity can be observed at $\text{pH} \leq 6$ in the CaCl_2 -free emulsions of the higher triglycerides (Fig. 1, VII—IX), this tends to show that a special inhibition asserts itself here. The phenomenon is not seen in the triglyceride experiments I—IV, where rather easily soluble acids are formed. As the addition of CaCl_2 in VII—IX more especially increases the activity in the pH range 5 to 6, it is reasonable to assume that the inhibition is due to the higher fatty acids and that their influence is abolished when they are converted into insoluble calcium soaps. That the higher fatty acids inhibit the action of pancreatic lipase appears from Fig. 2, in which are given the results of some experiments at different pH with tripropionin (A) without and with addition of 0.1 millimol lauric acid and tricaproin (B) without and with addition of 0.1 millimol lauric acid or stearic acid. These experiments show a beginning inhibitory effect at pH

values smaller than about 7, whereas no inhibition can be demonstrated in alkaline range. It ought to be mentioned that the inhibitory effect does not correspond to an unspecific accumulation of fatty acids. The addition of 0.1 millimol of the acid corresponding to the lower triglycerides does not inhibit the hydrolysis of tripropionin and tributyryl.

It is reasonable to assume that the inhibition is caused by the undissociated higher fatty acid, whereas the anion is inactive. Critical Tables gives pK for the fatty acids up to caprylic acid as about 4.8. From the experiments by HOLWERDA, VERKADE and DE WILLIGEN (1937) it is possible to compute a pK value of 5.1 for capric acid and undecylic acid. There is no reason to assume that further introduction of CH_2 -groups into the chain of fatty acids will change pK appreciably, and the inhibitory effect of the higher fatty acids can thus only be stated in the pH range where they are present partly in the undissociated form. As no hydrolysis can be found in more acid medium than $\text{pH} = 6$ in experiments VII—IX (without CaCl_2) even very small amounts of lauric acid, myristic acid and stearic acid are able to inhibit the enzymatic splitting of these triglycerides.

The pH-activity range for the enzymatic hydrolysis — both with and without CaCl_2 — of triolein (VI) is somewhat narrower than in the case of the saturated triglycerides. The special activation by CaCl_2 in acid range is less pronounced for triolein and tricaprin (V) than in the case of VII—IX.

Finally, we may point to the fact that the results of our experiments do not support WILLSTÄTTER's theory of the activation of lipase by the formation of complex adsorbates. In the first place the activation in the experiments I—IV, where no insoluble calcium soaps are formed, is of the same order of magnitude as in the rest of the experiments, where very slightly soluble calcium soaps are formed. Further some experiments given in Table I show that addition of calcium salts of oleic-, lauric- and stearic acid to the corresponding triglycerides is without any activating effect. These experiments were carried out at optimum pH for the different triglycerides.

In a previous paper (SCHÖNHEYDER and VOLQVARTZ, 1944) it has been shown that CaCl_2 does not influence the affinity between pancreatic lipase and the lower triglycerides in homogeneous solution. Experiments in the present paper indicate that the activation of pancreatic lipase towards the higher triglycerides in acid me-

dium is essentially due to the removal of strongly inhibiting fatty acids as calcium soaps. At the moment it is not possible to give a final explanation of the activation of lipase towards lower triglycerides, but nothing seems to run counter to the assumption that the mechanism is the same here as in the alkaline range in experiments with higher triglycerides.

Table 1.

The Hydrolysis of Some Triglycerides with Addition of the Corresponding Calcium Soaps at Optimum pH.

Triglyceride 0.283 millimol/30 cc system	Amount of Ca-soaps added, g	v
Triolein	0	10.7
	0.0934	11.2
Trilaurin	*0.2802	11.5
	0	12.6
Tristearin	0.0685	13.4
	*0.2059	11.6
Tristearin	0	6.8
	0.0949	7.15
	*0.2841	7.1

v = number of drops 0.1 n NaOH per 10 min.

* amount of Ca-soap equivalent to 50 mg CaCl₂.

Summary.

The activating influence of calcium chloride on the hydrolysis by pancreatic lipase of several saturated triglycerides and triolein has been investigated at different pH.

- With increasing number of carbon atoms in the fatty acids of the triglycerides the optimum pH for pancreatic lipase is displaced from 7 to 8.8.
- Addition of CaCl₂ does not alter the optimum pH for the enzymatic activity towards a triglyceride.
- CaCl₂ activates the hydrolysis of all triglycerides investigated in homogeneous as well as in heterogeneous systems and both in alkaline and acid medium.
- In the presence of CaCl₂ the pH-activity range for the higher saturated triglycerides is widened one pH unit on the acid side.
- The higher free fatty acids are supposed to depress the hydrolysis by pancreatic lipase in a high degree, and the pronounced activation by CaCl₂ in experiments with higher trigly-

erides is explained by the removal of free acids as insoluble calcium soaps.

6. The experiments do not support WILLSTÄTTER's theory of the activation of lipase by formation of complex adsorbates.

This work has been aided by a grant from the "Christian X's Fond".

References.

HOLWERDA, K., P. VERKADE and A. DE WILLIGEN, Rec. trav. chim. 1937. *56*. 82.
PEKELHARING, C. A., Hoppe-Seyl. Z. 1912. *81*. 355.
SCHÖNHEYDER, F., and K. VOLQVARTZ, Enzymologia 1944. *11*. In press.
SCHÖNHEYDER, F., and K. VOLQVARTZ, Acta physiol. scand. 1944. *7*. 376.
SCHÖNHEYDER, F., and K. VOLQVARTZ, Ibidem 1945 *9*. 57.
WILLSTÄTTER, R., E. WALDSCHMIDT-LEITZ and F. MEMMEN, Hoppe-Seyl. Z. 1923. *125*. 93.
WILLSTÄTTER, R., and E. WALDSCHMIDT-LEITZ, Ibidem 1923. *125*. 132.

From the Biochemical Institute, Aarhus University, Denmark.

Studies on Serum Phosphatase Activity in Relation to Experimental Biliary Obstruction in Rabbits. I.

By

OLAF JALLING, THOMAS LAURSEN and KIRSTEN VOLQVARTZ.

Received 4 May 1945.

A number of theories have been suggested as to the mechanism of the increase in serum phosphatase activity in obstructive jaundice first described by ROBERTS (1930). The most common theories can be summarized in the following way. 1) The hyperphosphatasemia (hph) is due to retention of phosphatase which is normally excreted by the liver in bile. This theory is advanced by ROBERTS (1930) and other investigators in this field have supported his view. The main argument for this theory is that phosphatase has been found in bile. Some authors e. g. SCHIFFMAN and WINKELMANN (1939) maintain that the hepatic function is of great importance to the degree of hph. 2) THANNAUSER, REICHEL, GRATTAN and MADDOCK (1937) have suggested that the hph is due to an activation of the normal serum phosphatase rather than to an actual increase in the amount of circulating phosphatase and CANTAROW (1940) seconds their opinion. 3) A third theory has been put forward by AUSTONI and COGGI (1934). According to these authors the hph is due to absence of bile in the intestine which presumably is followed by disturbance in the calcium-phosphorus metabolism. AUSTONI and COGGI stress that biliary fistulae in human and dog are followed by hph.

It is hardly possible to elucidate the mechanism of the increased serum phosphatase by biliary obstruction by means of clinical observations, and experiments on dogs have not finally solved

this question. As hph has been found to be pronounced in rabbits after ligation of the common bile duct and as these animals are cheap they were used as experimental animals in this work. The hph in rabbits is not due to trauma of the common bile duct or to an activation of the normal serum phosphatase. In bile fistulae rabbits no increase in the serum phosphatase activity can be stated, this operation is rather followed by a decrease in the serum phosphatase activity. The increase may partly be explained by retention of phosphatase which is normally excreted with the bile but besides a hyperproduction of phosphatase in one or more organs with delivery to the blood must play a rôle.

Methods.

In all experiments white rabbits have been used. The average weight of the animals has been about 2 kg. The animals were kept in individual cages. The diet has been kohlrabi and hay. The animals were kept on the diet for about a week before experimentation. The operations were carried out with aseptic precautions. None of the animals operated on ate during the first 48 hours after the operation.

Serum phosphatase activity was estimated by the method of BUCH and BUCH (1939). 2 to 3 ml of blood were drawn for each determination. For the estimation of the amount of phenol liberated Pulfrich's photometer was used instead of a universal colorimeter. Filter S 72 and 0.5 cm cuvette were employed. The measurements should be carried out not later than one hour after the development of the colour (5 to 60 min. after the addition of Na_2CO_3). In the case of very small phosphatase values 1 cm cuvette ought to be used. By means of a standard curve (Fig. 1) constructed on the basis of known phenol solutions the

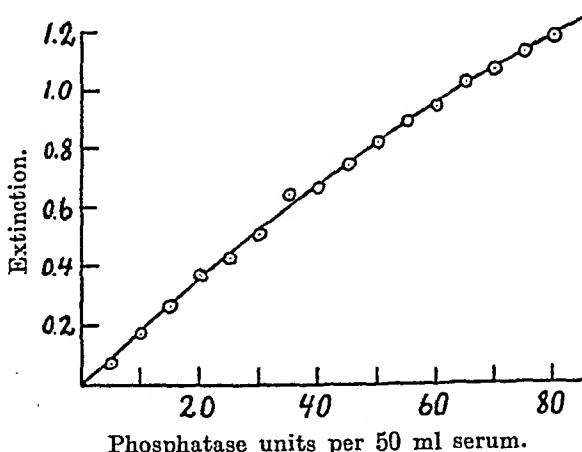


Fig. 1. Standard curve for colorimetric phosphatase determination with the Pulfrich photometer.

extinction read is converted into phosphatase units (phu). It was convenient to adopt as a unit of activity the amount of enzyme capable to liberate from di-sodium phenylphosphate 1 mg of phenol during the first 15 min. of the hydrolysis at 37.5° C and pH 9.6. The phosphatase activity of a sample is given as number of phosphatase units (phu) per 50 ml serum. This unit equals Buch's unit. The coefficient of variability of a duplicate determination was found to be 2.7 per cent. BODANSKY (1932) has in human serum demonstrated an increase in phosphatase activity of serum which was left to stand for 24 hours. The authors have made a similar observation on rabbit serum, a rise of 11 per cent being found after 24 hours. All the phosphatase determinations were therefore carried out within one hour of blood-drawing.

Experimental.

Normal Material. The distribution of serum phosphatase units in normal rabbits (46 different animals) is illustrated by the probit method in Fig. 2. It is seen that the distribution is practically

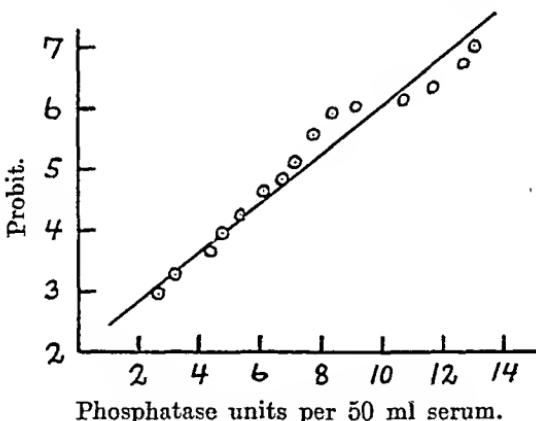
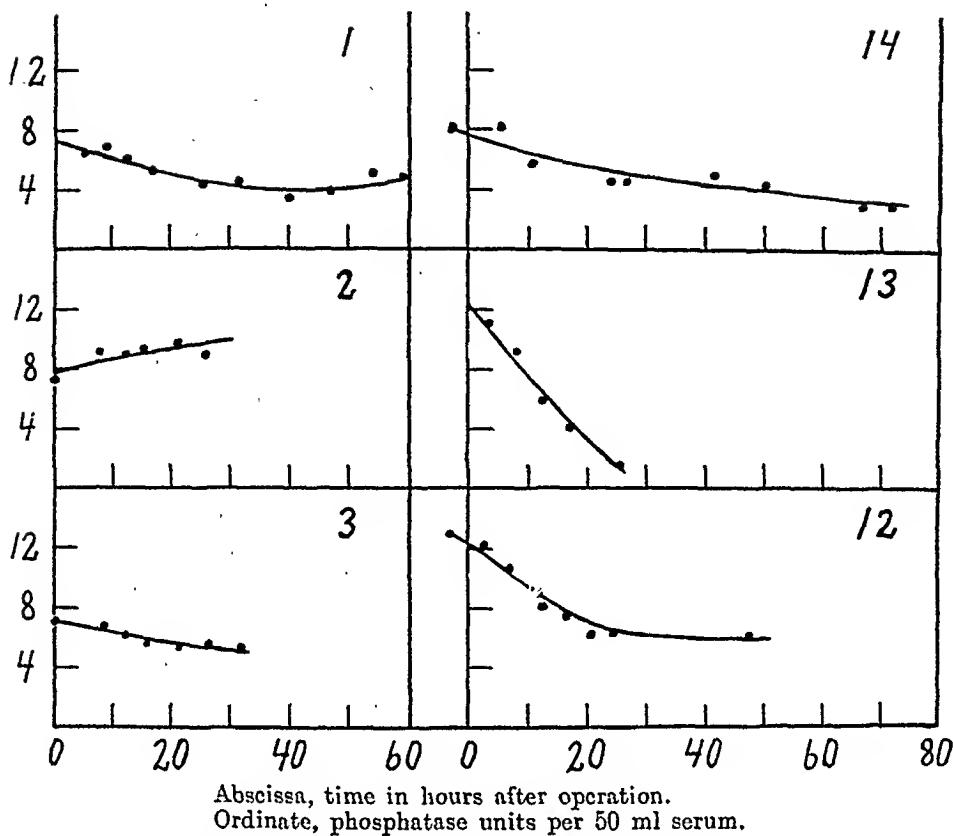


Fig. 2. The relation between probit and phosphatase units per 50 ml serum in 46 normal rabbits.

normal. The average value found is 7.4 phu. From the inclination of the straight line $\sigma = 2.5$ phu is computed. When blood is drawn at intervals of some days the serum phosphatase activity in normal rabbits does not vary very much. On inanition and bleeding 6 to 7 times during a 40 hour period in two cases out of three a moderate drop in the serum phosphatase activity could be stated (Fig. 3). BODANSKY and JAFFE (1931) and JØRGENSEN (1944) made the same observation on fasting rabbits.

Obstruction of Common Bile Duct. This condition was produced in 5 animals. All rabbits showed a marked elevation in serum phosphatase (Fig. 4). The increase occurs shortly after operation



Abscissa, time in hours after operation.
Ordinate, phosphatase units per 50 ml serum.

Fig. 3. Effect of inanition and bleeding on serum phosphatase activity in normal rabbits (Rabbits 1 to 3), and serum phosphatase activity in bile fistula rabbits (Rabbits 12 to 14).

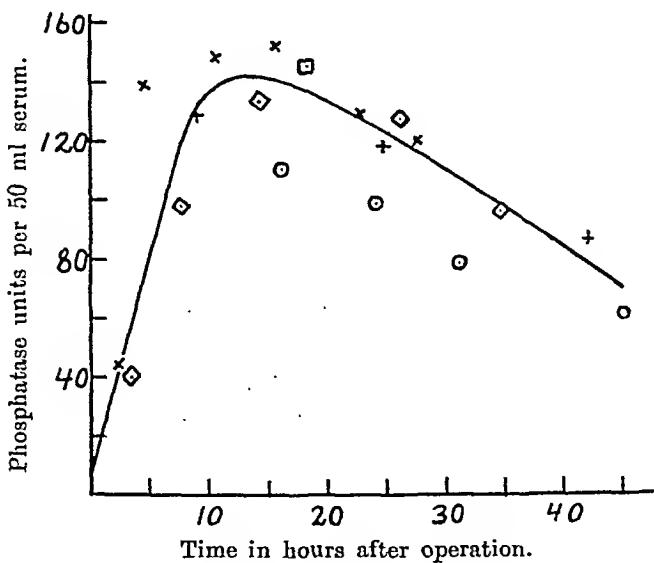


Fig. 4. The variation in serum phosphatase activity in 5 rabbits after ligation of the common bile duct.

reaching a maximal value of 140 phu in about 15 hours followed by a slow fall. By frequent bleeding during the first 24 hours the time of survival was shortened to about 40 hours. In one animal blood was only drawn once a day. In this animal the maximal value found was 146 phu and 240 hours after operation a value of 17.7 phu was found.

Anastomosis between Gall Bladder and Duodenum. That the hph found in rabbits with ligation of the common bile duct is not due to a mechanical action on the common bile duct appears from the following experiments. In three rabbits an artificial connection through a thin rubber tube was produced between the gall bladder and duodenum; then the common bile duct was ligated.

Table I.

Variations in Serum Phosphatase Activity after Anastomosis between Gall Bladder and Duodenum in Rabbits with Ligation of the Common Bile Duct.

Rabbit 9		Rabbit 10		Rabbit 11	
t	Ph	t	Ph	t	Ph
0	6.4	0	7.1	0	7.1
6 ¹ / ₂	6.7	2 ¹ / ₄	8.6	8	5.6
10	5.8	18 ¹ / ₄	4.5	18	4.4
13	5.9	23	4.6	22	8.0
18 ¹ / ₂	5.1	40	8.0	26 ¹ / ₂	7.2
25	6.0				

t refers to hours after operation,

Ph refers to phosphatase units in 50 ml serum.

As can be seen in Table I the small decrease found in controls is not observed, but it is certainly not justified to state an increase in serum phosphatase activity following the operation.

Phosphatase Activity in Mixtures of Normal and Pathological Serum. In order to check the activation theory that has been advanced by THANNHAUSER and coworkers some experiments were performed in which phosphatase activity was determined in serum with normal and enhanced activity as well as in mixtures of these sera. The results of this investigation are shown in Table II. It appears from this table that in all cases a good agreement between observed and calculated phosphatase values was found.

Bile Fistulae Rabbits. hph is described in dog and in human in the case of gall fistula (FREEMAN, CHEN and IVY, 1938, THANN-

Table II.

The Phosphatase Activity in Mixtures of Normal and Pathological Serum.

Composition of mixtures	Phosphatase units per 50 ml serum	
	Found	Calculated
1 A + 0 B	6.20	6.20
0 A + 1 B	53.05	53.05
1 A + 4 B	43.60	43.68
1 A + 2 B	37.75	37.43
1 A + 1 B	29.65	28.63
2 A + 1 B	22.50	21.82
4 A + 1 B	15.90	15.57

A refers to normal serum; B refers to pathological serum.

HAUSER, REICHEL, GRATTAN and MADDOCK, 1937, MORRIS and PEDEN, 1937). This finding has been a stumbling block against the retention theory. In what follows the response of rabbits to bile fistula has been examined. In 3 rabbits the common bile duct was ligated, whereupon a rubber tube was inserted into the gall bladder. Through this drain the bile was collected continually, its volume and phosphatase activity being measured. From the results given in Table III the amount of phosphatase excreted

Table III.

*Amounts of Phosphatase and Volume of Bile
in Three Fistula Rabbits.*

Rabbit 12			Rabbit 13			Rabbit 14		
t	a	Ph	t	a	Ph	t	a	Ph
3/4	9	49.8	3 1/2	27	6.9	1/4	2	31.2
1 1/4	7		8 1/4	54	4.6	4 1/2	46	3.6
2 1/4	6.7		12 1/2	27	7.5	9	35	2.2
6 1/4	23	7.7	17	21	10.3	13 1/2	24	5.5
7 3/4	11.7		25 1/4	15	8.3	17	18	6.6
8 3/4	7.5	10.7				25 1/2	26	7.1
10	10.5							
11	6							
12	7.5	12.7						
17 3/4	35	17.9						
24	38	32.6						

t refers to hours after operation,

a refers to bile in ml,

Ph refers to phosphatase units in 50 ml bile.

during the first 15 hours can be computed approximately. Rabbits 13 and 14 excreted 17.1 and 11.2 phu respectively during 17 hours. In the case of Rabbit 12 it is more difficult to calculate the amount of phosphatase excreted with the bile because phosphatase determinations were not carried out on all the bile samples. Using average values of phu per ml (computed as the mean of phu per ml in the preceding and following sample) in the samples where phosphatase determinations were not carried out it was found that Rabbit 12 has excreted 42.5 phu during $17\frac{3}{4}$ hours. The serum phosphatase activities during the experimental period are given in Fig. 3.

Discussion.

From the experiments it seems apparent that in rabbits a prompt and marked elevation of serum phosphatase activity occurs in response to ligation of the common bile duct. These animals are therefore suitable for studies on the mechanism of the development of hyperphosphatasemia. hph is not caused by a reflectory release from the common bile duct and the increase in serum phosphatase activity is a real rise of phosphatase.

In bile fistula rabbits no hph but rather a fall in the serum phosphatase activity is observed, this fall being somewhat larger than in fasting controls from which the same amount of blood was taken as in the case of the experimental animals. According to the retention theory hph is due to retention of the phosphatase normally excreted in bile. Regardless of the origin of the phosphatase present in bile a moderate fall is quite natural, when the retention of bile components causes hph, and from the findings in rabbits one may suppose that the rise found in dog and human with bile fistula is hardly caused by bile loss. That absence of bile in the intestine is of no importance in the production of hph appears also from the fact that in spite of persistent complete obstruction a fall in serum phosphatase activity occurs. Some preliminary experiments not presented here showed that the degree of hph is not influenced appreciably by the administration of bladder-gall from rabbits to animals with complete obstruction of the common bile duct.

The experiments in which the phosphatase in bile and the amount of bile produced during a certain time were determined, may shed light on the problem whether the hph can be explained as a simple consequence of transition to the blood of the phosphatase.

tase normally excreted with the bile. If all the amount of phosphatase which is measured during the first 17 to 18 hours were retained in the blood, then the increase which can be calculated, blood volume (5 per cent) and hematocrit (33 per cent) being considered, would be much smaller than the value observed 15 to 20 hours after ligation of the common bile duct. hph can thus not be explained by retention alone wherefore it seems reasonable to assume that a hyperproduction in one or more organs takes place. Experiments on this problem will appear in a following paper.

Summary.

The problem concerning the mechanism of the development of hyperphosphatasemia in obstructive jaundice has been taken up for renewed examination on rabbits, which have been found suitable for such experiments.

1. The increase in serum phosphatase after ligation of the common bile duct starts immediately and reaches a maximal value 15 to 20 times greater than the normal value in about 15 hours, followed by a slow drop during the next days.
2. The increase in the serum phosphatase is a real rise in the amount of phosphatase.
3. The increase in serum phosphatase is not released from the common bile duct.
4. The results of the experiments do not support the "activation theory". Nor can they be explained by the absence of bile in the intestine.
5. The retention of bile phosphatase alone cannot explain the hyperphosphatasemia.

References.

AUSTONI, B. and B. COGGI, Pr. méd. 1934. 42. 1594.
BODANSKY, A., Proc. Soc. Exp. Biol. N. Y. 1932. 29. 1292.
BODANSKY, A. and H. L. JAFFE, *Ibidem* 1931. 29. 199.
BUCH, I. and H. BUCH, Acta med. Scand. 1939. 101. 211.
CANTAROW, A., Amer. J. clin. Path. 1940. 10. 858.
FREEMAN, S., Y. P. CHEN and A. C. IVY, J. Biol. Chem. 1938. 124. 79.
JØRGENSEN, G., Undersøgelser over Serumfosfataser. Disp. København 1944.
MORRIS, N. and O. D. PEDEN, Quart. J. Med. 1937. 30. 211.
ROBERTS, W. M., Brit. J. Exp. Path. 1930. 11. 90.
SCHIFFMAN, A. and L. WINKELMANN, Arch. Int. Med. 1939. 63. 919.
THANNHAUSER, S. J., M. REICHEL, F. GRATTAN and S. MADDOCK, J. Biol. Chem. 1937. 121. 727.

From the Biological Institute of the Carlsberg Foundation,
Copenhagen.

Oxygen Supply and Tissue Growth in vitro.

By

ALBERT FISCHER, BØRGE FOGH and AAGE BOHUS JENSEN.

Received 7 May 1945.

1.

It is known that the peripheral cells of the fibroblast culture degenerate much sooner than the closely packed cells in the central part; the latter cells preserve their fresh, normal appearance, as may be seen from regeneration experiments (FISCHER (1930)). It is also known, that the peripheral cells of the culture towards the end of the cultivation period become irreversibly damaged and hardly can be brought to divide, at the time the degeneration sets in. Apparently it is impossible to modify this condition by washing or supplying fresh nutrient substance, when the cells have reached the stage when they are filled with fat granules. Even ample amounts of the nutrient substances cannot prevent the occurrence of this degeneration. It has, on the other hand, been possible to preserve the cells of the central part of the culture in a state of good condition for more than a year (without transfer to a new medium) by means of ample supply of nutriment and by washing (PARKER (1936)).

The distribution of the mitoses is likewise characteristic. There are practically no mitoses in the central part of the culture, the mitotic division activity being localized to a broad marginal zone (FISCHER and PARKER (1929); JACOBY (1937)).

The structure of the fibroblast culture is characteristic with respect to cell density. The central part of the culture is numerous cell layers in thickness and the cells are closely packed; from here the cell density diminishes radially towards the marginal zone

where the thickness of the culture only is that of a single cell layer and where the density is small; in the periphery of the culture the cells are more or less scattered, more so when the migration is about to cease.

It must be assumed that the wide variations in cell concentration and tissue thickness make the living conditions very different in the marginal and in the central zone of the culture. The distribution of the mitoses, together with the fact that the peripheral cells show fat degeneration much sooner than the closely packed cells in the central part may presumably be regarded as expressions for this fact.

Little is known as to the causes of these phenomena, but one may, as an introduction to an analysis of the problem, assume that the reason for these circumstances might be found in the difference in the oxygen tensions in the particular sections of the culture. The wide difference between cell density and tissue thickness in the central and in the marginal zone must give rise to differences in the consumption of oxygen and the conditions governing the supply with oxygen. The present investigation deals with these problems.

In different ways we tried to determine the lowest value of the oxygen tension necessary in the culture chamber if the cells in the thickest and densest tissue mass of the culture are to have their normal oxygen consumption satisfied. The experiments involved the use of a vital stain as indicator for the presence of oxygen.

The staining was done by means of brilliant cresyl blue (GRÜBLER) which in the oxidized state is blue and in the reduced state colourless. In the main we have followed the technique of GOTZWALT FISCHER and HARTWIG (1937) to which reference is made for further details.

The staining was carried out under anaerobic conditions with the leuco-base of the dye, since this, according to the authors mentioned, gives a better penetration into living cells. The stock solution of the dye (1 : 1,000 in distilled water) was diluted by means of Ringer-glucose (0.15 per cent of glucose) to protect the culture medium against any washing out of sugar. The leuco-base (concentration 1 : 5—6,000) was allowed to act on the hanging drop culture of the coverslip for 20 to 30 minutes at a temperature of 25—30° C. Then the culture was transferred to fresh Ringer-glucose to which the air had free access, whereby the dye in the tissue was oxidized and assumed a blue colour. After 15—30 minutes the cultures were suitable for the experiments.

The coverglass containing the cultures were stuck on to the lids of Gabritchewski dishes by means of a little vaseline — two on each lid.

In the preliminary experiments some basic solution of pyrogallol was placed in the marginal trough of the Gabritchewski dish, and in the middle of the dish a little Ringer solution to prevent the drying out of the culture.

After the lid had been replaced on the dish (with apertures open) nitrogen (having first passed over red-hot copper) or hydrogen was led through the dish for ca. 20 minutes, whereupon the lid was turned to close it tight. In this way we attained an effective removal of the oxygen in the culture chamber, resulting in a decoloration of the tissue. The Gabritchewski dish was placed on a microscope stage which was surrounded by a heated box wherein the temperature was maintained at 30—35° C.

In the experiments designed to determine the oxygen tension necessary for the requirement of the tissue, gas mixtures of varying oxygen content (checked by analysis) were led through the Gabritchewski dishes for about 30 minutes, whereupon the dishes were closed. One or two newly stained cultures were used for each experiment. Experiments were made with ordinary commercial nitrogen, as well as with nitrogen free of oxygen. In each case the nitrogen was led through a 2 per cent solution of bicarbonate ahead of the Gabritchewski dish. The other gas mixtures which had a higher oxygen content were estimated and prepared from a mixture of air and nitrogen (ordinary commercial product) and kept over a 2 per cent bicarbonate solution in a gasometer; the contents of oxygen and carbon dioxide were checked by analysis. The mixtures were led through the Gabritchewski dishes for about 30 minutes, whereupon the dishes were closed and the observations made.

Coverslip cultures of ordinary 7—15 passages old fibroblasts from the hearts of 7—9 days old chick embryos were used. The culture medium was only slightly stained by the dye used, so it was easy to observe the colour of the cells.

In the past, several experiments have been made with the staining of embryonic organisms by means of various oxidized dyes, observations being made of the rate at which the various tissues were able to reduce these dyes under anaerobic conditions (CHILD (1937), RULON (1935), GOTTWALT FISCHER and HARTWIG (1937), PIEPHO (1938)). The experiments have shown that the tissues reduce the dyes at widely different rates under the conditions stated. Since these experiments deal with a comparison of tissues which undoubtedly are very different in nature (widely different developmental potentialities) the observed differences in the rate of reduction must no doubt represent inherent characteristics. The case is somewhat different with regard to our investigation. Here the cells must be assumed, throughout the whole culture, to be essentially alike. The differences in the rate

at which the cells reduce the absorbed dye under the oxygen tensions prevailing in the culture chamber must be assumed to be occasioned by the different conditions governing the supply of oxygen, as well as by differences in the amount of oxygen consumption owing to variations with respect to cell density and thickness of tissue.

In preliminary experiments observations were made on the decolourizing phenomena of stained cultures placed in an atmosphere of diminishing oxygen tension. It was found that the central part of the culture was decolorized quite rapidly, while the peripheral zone did not lose its colour until some time had passed; the most peripheral cells retained their colour for the longest time. If at this stage air was led into the culture chamber the stain reappeared. Then the decoloration of the tissue could be observed once more.

The observed decoloration must be due to the circumstance that in part of the culture a deficiency of free oxygen is created, which causes the cells to use the oxygen bound in the dye; with diminishing oxygen tension this occurs first in the central part of the culture.

2.

In order to understand the living conditions in the culture it is necessary to consider its topography.

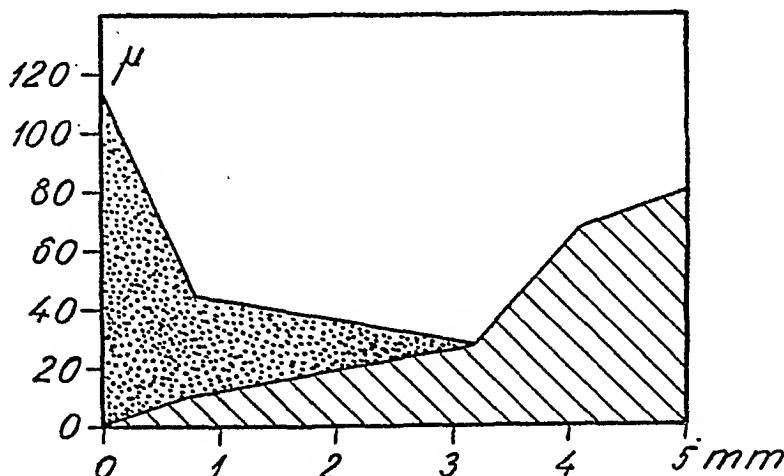


Fig. 1.

Fig. 1 shows a cross section from the centre to the edge of the tissue fragment in a 48 hours' old coverslip culture. The

thickness of the tissue and the plasma coagulum is drawn to a scale 25 times larger than that used for the diameter of the ground area of the tissue. The cross-hatched area represents the tissue mass of the culture, and the dotted area above represents the plasma coagulum which immediately after the transfer to a new medium covers the culture with a thick layer, and which to a large extent retracts over the tissue owing to the squeezing out of fluid from the coagulum (syneresis) caused by the activity of the cells; the result of this retraction is a distinct depression in the culture medium. This depression is in all cases filled with serum squeezed out of the culture medium. — During measurement the surface of the coagulum was set off by means of scattered Lycopodium spores, the fluid drop first being removed with the aid of filter paper.

The figure shows that the thickness of the tissue varies greatly from centre to margin.

Table 1.

Culture No.	The thickness of the plasma coagulum above the marginal cells, in μ	The thickness of the central part of the tissue culture, in μ
7647.....	244	107
7656 ₁	104	65
3570 ₂	112	80
7507 ₁	224	81
3449 ₁	133	148
3442 ₁	177	66
4820.....	116	80

Table 1 records the thickness of the central part of a number of cultures; it is seen that there is great variation from one culture to another. The thickness of the peripheral part is very constant, *viz.*, a few μ , the explanation being that the extreme marginal zone always is one cell-layer thick.

Our measurements show finally (see Table 1) that the thickness of the layer of plasma coagulum which covers the marginal zone of the culture is very considerable and also very variable. Some of the cultures undoubtedly lacked plasma coagulum over the central part as seen in Fig. 1. It is obvious that these differences in tissue thickness must produce corresponding differences in the amounts of oxygen necessary to satisfy the oxygen consumption in the various parts of the culture. The vital staining experiments

show that when the oxygen tension in the culture chamber falls, there soon occurs an oxygen deficiency in the central part of the culture which is thick and rich in cells, while oxygen deficiency in the peripheral, scattered tissue cells does not occur until much later, and apparently at a much lower oxygen tension.

With these observations as starting point it should be possible to determine the oxygen tension at the very moment a noticeable decoloration of the central part of a culture occurs. By leading nitrogen free of oxygen through the culture chamber we obtained a more or less distinct decoloration of the central part of the cultures (most pronounced macroscopically). No decoloration occurred in the marginal zone of the culture. When the culture chamber was opened the central part of the tissue resumed its original colour. When nitrogen (found by analysis to contain 0.87 per cent of oxygen) was led through the chamber the central part of the culture was likewise distinctly decolourized. When the gas mixture contained 1.94 per cent of oxygen the decoloration of the central parts was not so distinct, though still clearly discernible, but when the oxygen content was further increased no decoloration occurred.

It is undoubtedly a fact, however, that when no decoloration occurs at oxygen contents higher than about 2 per cent, this does not prove that the oxygen supply actually is adequate for the oxygen consumption of the whole central part of the tissue. One should remember, for one thing, that by the present method it is hardly possible to recognize a decolouration of the deepest lying layers of the tissue mass, *i. e.*, those closest to the coverslip, since this mass is covered by a thick or thin layer of stained cells. Moreover, our experiments have for unknown reasons yielded a rather poor staining of the central part of the cultures, a fact which influences the perception of differences in decoloration. Hence we must assume that the above mentioned value is too low, and we have therefore by other means tried to obtain a rough estimation of the oxygen tension which is required if the whole tissue mass is to be supplied with oxygen.

3.

A series of determinations were made of the oxygen consumption of excised tissue cultures at different oxygen tensions, the idea being that with diminishing oxygen tension a point would

be reached where this pressure would be insufficient, under the prevailing conditions of diffusion, to provide the central part of the tissue with the necessary oxygen supply, for which reason the oxygen consumption during continued diminution of the oxygen tension would decrease.

Back of this idea is the well known fact (WARBURG (1926)) that the oxygen consumption within wide limits is independent of the oxygen tension, and that changes in the conditions of diffusion must be the sole cause of the changes in the magnitude of the oxygen absorption.

The cultures used were cut in the manner used when a tissue is to be transferred to a new medium; each experiment involved about 20 cultures which were placed in a Warburg chamber containing a Ringer-glucose solution (0.2 per cent) to which a few drops of serum had been added.

Since no determination was made of the mass of the tissue, the determinations of the oxygen consumption at different pressures had to be made on the same set of cultures. First a determination was made at an oxygen content equal to that of air, next at one or two lower oxygen tensions, followed by a determination in air in order to establish whether the respiratory intensity had changed since the beginning of the experiment.

The tools used in these experiments were not sterile, but controls in air had proved that the infection had not reached a magnitude that made any decisive difference in the respiratory intensity of the tissue.

It was found that the oxygen consumption of the tissue within each set of cultures was constant until the oxygen tension had fallen to about 4 per cent. This must mean that the central parts of the tissues at oxygen tensions about this percentage or lower are insufficiently supplied with oxygen for the satisfaction of the usual oxygen consumption.

Since the cultures during the respiratory measurements were floating freely in the medium and therefore received oxygen from both sides, an oxygen tension of about 8 per cent was necessary for the supply of the deepest lying parts of a culture attached to a coverslip since the oxygen supply in this case would occur from one side only. For the reasons previously cited this value, which is considerably higher than the one found in the vital staining experiments, must be regarded as the most reliable one.

4.

In the coverslip culture the volume of the culture chamber is, from measurements, about 500 mm³. Of this space, 100 mm³ are occupied by oxygen at the oxygen tension 0.2 atmosphere, which prevails at the beginning of the cultivation.

According to LIPPMANN (1932) the oxygen consumption of an ordinary heart fibroblast culture may be put at 22 mm³ oxygen in the course of a cultivation period lasting 96 hours, the experimental conditions otherwise being the same as in our vital staining experiments. This means that tissue cultures may be estimated to consume $\frac{1}{4}$ of the available oxygen, and that the oxygen tension after 96 hours has fallen to 0.15 atmosphere.

One will notice that it is possible to use up to one-half of the oxygen which may be estimated as being available, before the oxygen tension reaches a value so low that the central part of the tissue culture receives so little oxygen that the oxygen consumption falls off. Since normally about $\frac{1}{4}$ of the available oxygen is consumed, sufficient oxygen will be present to satisfy the consumption of the cells in all parts of a culture. It is perhaps possible that oxygen deficiency may occur in the case of very thick tissue cultures towards the end of a cultivation period.

The variation in the oxygen tension which presumably occurs in the tissue during a cultivation period falls, in all essentials, within the range where the oxygen consumption of the cells is not affected by the oxygen tension. If the oxygen tension then should have any influence on the metabolism of the cells, and thereby on their state of preservation in the different parts of the culture, it must be assumed that this phenomenon is independent of the oxygen consumption, and thus a matter of a direct or indirect effect of the oxygen tension on other processes in the cell body (cf. LASER (1937)); we have no actual support for this idea, however.

The conclusion may be drawn that the absence of mitoses in the central part of a culture under normal conditions cannot be attributed to lack of oxygen since the amount of oxygen present in the tissue is sufficient for normal oxygen consumption. Nor can the degeneration phenomena which one observes in the marginal zone of a culture be due to oxygen deficiency. The plasma coagulum has a rather considerable thickness above the marginal cells, our measurements showing values varying from 104 to

244 μ . The conditions governing the supply of oxygen are determined by the conditions of diffusion in the column of plasma coagulum above the marginal cells, and are moreover dependent on the oxygen tension in the culture chamber and on the oxygen consumption of the marginal cells. The latter, however, cannot amount to much per unit of surface of the one cell layer thick marginal zone, where the cells moreover are scattered; add to this that the marginal zone must have a rather considerable oxygen supply from the free culture medium outside the edge of the tissue (LIPMANN (1932)). There can be no doubt that the cells of the marginal zone under normal conditions will receive enough oxygen to cover their normal consumption.

It is also possible to prove directly that the oxygen tension has no influence on the rate at which the degeneration progresses in the cells of the marginal zone. Heart fibroblasts were cultivated in Carrel flasks in a gas mixture of 95 per cent oxygen and 5 per cent carbon dioxide. For control of the reaction of the medium a drop of phenol red was added to the fluid phase so that the concentration in the medium became 0.005 per cent. Heart fibroblasts were also cultivated in an atmosphere of pure nitrogen. Finally, control cultures were made with air in the flasks. No essential difference was found in the setting in of the degeneration in the marginal zone of the control and the experimental culture.

Our experiments have furnished no explanation of why the cells are preserved so well in the central part of the culture.

Summary.

1. A determination was made of the oxygen tension which is necessary if the mass of the whole tissue culture is to be supplied with just enough oxygen to satisfy the normal demand of the cells.

2. On the basis of the knowledge of the oxygen consumption of a culture and the amount of oxygen which is available at the beginning of the cultivation it is possible to demonstrate that enough oxygen must be present to satisfy the supply for all parts of the culture.

3. It is apparent that the influence of the oxygen tension cannot be responsible for the very large difference observed in the rate at which the cells degenerate in the closely packed central

part and in the marginal zone. Nor does it seem possible that low oxygen tension in the central tissue fragment can account for the discontinuation of the mitotic division activity in the central part of the culture.

Thanks are due to "Kong Christian den Tiendes Fond" and "P. Carl Petersens Fond" for grants in aid of this work.

Literature.

CHILD, C. M., Arch. Entw. Mech. Org. 1937. *135*. 426.
EPHRUSSI, B., L. CHEVILLARD, A. MAYER and L. PLANTEFOL, Ann. Physiol. Physicochim. biol. 1929, *5*. 642.
FISCHER, A. and R. C. PARKER, Brit. J. exp. Path. 1929. *10*. 312.
GOTTWALT FISCHER, F. and H. HARTWIG, Z. vergl. Physiol. 1937. *21*. 1.
JACOBY, Fr., Arch. exp. Zellforsch. 1937. *19*. 241.
LASER, H., Biochem. J. 1937. *31*. 1671.
LIPMANN, F., Biochem. Z. 1932. *244*. 177.
LIPMANN, F., Biochem. Z. 1933. *261*. 157.
PARKER, R. C., J. exp. Med. 1936. *64*. 121.
PIEPHO, H., Biol. Zbl. 1938. *58*. 90.
RULON, O., Protoplasma, 1935. *24*. 346.
WARBURG, O., Biochem. Z. 1923. *142*. 317.
WARBURG, O., Über den Stoffwechsel der Tumoren, Berlin 1926.

From the Laboratory of Zoophysiology, Copenhagen University.

The Osmotic Concentration of Human Lacrymal Fluid.

By

A. KROGH, C. G. LUND and K. PEDERSEN-BJERGAARD.

Received 30 May 1945.

In Pharmacopoea Helvetica 1933, Pharmacopoea Gallica 1937, Dispensatorium Danicum 1934 and 1938 and finally in Pharmaconomia Svecica 1943 it is stated that solutions for instillation into the eye should be isotonic not with blood ($= 9\text{ \%}\text{ NaCl}$), but with a $14\text{ \%}\text{ solution of NaCl}$; and statements that this corresponds to the osmotic concentration of tears are quite common in textbooks.

From a physiological point of view this statement appears improbable *a priori*, and it was considered desirable to test it experimentally before including it in the Dispensatorium Danicum now under revision. Lacrymal fluid was collected therefore from a small number of persons and the concentration estimated by the very sensitive and accurate vapour pressure method originally proposed by A. V. HILL and applied by us in the modification described by KROGH (1938). The material was collected in pyrex tubes similar to those described by WIDMARK for taking blood samples for alcohol determination. One end of the capillary was placed along the inner side of the lower lid drawn slightly downwards by means of a finger. This stimulus sufficed to initiate a sufficiently rapid secretion of tears. The sampling tube was sealed off at both ends and brought to the laboratory. The determination took place within 24 hours. Preliminary determinations on two subjects gave the result that the osmotic concentration corresponded to 9.1 and $9.2\text{ \%}\text{ NaCl}$ respectively.

On 5 men and 5 women the lacrymal fluid was compared with the serum of the same subject with the following results.

Sex	Age	Osmotic concentration in % NaCl	
		Serum	Lacrymal fluid
♂	41	8.9	8.9
♂	30	8.9	9.2
♂	32	8.7	9.0
♂	32	8.8	8.8
♂	28	8.7	8.9
♀	41	8.5	8.9
♀	31	8.1	8.7
♀	33	8.8	9.2
♀	30	8.5	9.0
♀	29	8.5	9.0
		Average 8.6	9.0

The average for the serum of women is lower than for men viz. 8.5 against 8.8, but the lacrymal fluids show no difference. It appears doubtful whether the slight average difference between the tears and the serum is real, since some slight evaporation from the eye surface is difficult to avoid.

We have tried to discover and control the source of the statement concerning the isotonicity of tears with 14 % NaCl and have traced it back to MASSART (1889) who studied the reactions of some lower organisms and certain tissues to varied concentrations of saline substances. His experiments on the human eye were carried out on two subjects by instilling drops of solutions of NaCl, Na₂SO₄, etc. at the nasal corner of the eye where the sensitivity is maximal according to him. The solutions were only slightly below body temperature. He states that hypertonic solutions produce sensations distinctly different from those caused by hypotonic and finds a rather sharp limit at about 250 mM in one subject and between 200 and 250 in the other. He figures out the average to correspond to 13.9 % NaCl.

MASSART's result is accepted by HAMBURGER in his great work: Osmotischer Druck und Ionenlehre III, 162, 1904 and supported by a chemical analysis by LERCH who finds 1.3 % NaCl while MAGAARD'S analysis gives the figure 0.42 %.

A Cl analysis carried out in the Zoophysiological Laboratory and calculated as NaCl gave 0.73 % in one subject and 0.81 % in another.

We have tried to repeat MASSART's observations first by instilling NaCl solutions as described by MASSART into the eyes of

4 subjects of which two were trained observers. One of the trained observers could distinguish between 6 %_{oo} NaCl and 20 %_{oo} which latter gave after several seconds a slightly disagreeable sensation. The other trained observer could even feel 15 %_{oo} as very slightly disagreeable, but the two untrained observed no difference whatever between the concentrations from 6 to 20 %_{oo}. On one of the trained subjects the tests were repeated using an eyebath kept on for at least one minute. By this technique NaCl solutions from 8 to 12 %_{oo} were found completely indifferent. 6 and 13 %_{oo} were just perceptibly disagreeable after almost a whole minute, but it was not possible to decide by the sensation whether they were hypo- or hypertonic. 15 %_{oo} was slightly disagreeable after nearly 1 minute and 20 %_{oo} definitely so after 1/2 minute.

The normal eye is no doubt rather insensitive to concentration differences, but the case may be different in disease, and there can be no reasonable doubt that fluids applied to human eyes for any length of time should be blood isotonic.

References.

KROGH, A., Osmotic regulation (p. 211). London 1938.
MASSART, J., Arch. d. Biol. 1889. 9. 537.

From the Biochemical Institute of the University, Aarhus, Denmark.

Chromatographic Method for the Determination of Glutamic and Aspartic Acid in Mixtures of Different Amino Acids.

By

SVEN DARLING.

Received 9 June 1945.

Methods for chromatographic separation and determination of amino acids have been outlined by TURBA (1941), TURBA and RICHTER (1942), WIELAND (1942) and WIELAND and WIRTH (1943). By these methods it is possible to separate the basic amino acids from the others, the former being adsorbed to fullers earth. By the use of aluminium oxide that has been previously treated with diluted hydrochloric acid or acetate buffer the aminodiearboxylic acids are strongly adsorbed, whereas the neutral and basic acids can be easily eluated with water. The dicarboxylic acids can be eluated together by means of diluted potassium or sodium hydroxide. When glutamic and aspartic acid are to be determined separately, the former is first eluated with acetic acid, then the latter with potassium hydroxide. The quantitative determination of the amino acids is carried out on the eluates according to TURBA by van Slyke's method after repeated evaporation in vacuum. WIELAND (1942) determines the neutral and basic amino acids by titration in alcohol of an aliquot part of the eluates after concentration in vacuum. WIELAND and WIRTH (1943) determine glutamic- and aspartic acid with ninhydrin. The blue colour developed is measured colorimetrically. This method is rapid but rather inaccurate. Common to these methods is the use of relatively large amounts of amino acids (5—25 mg) requiring large amounts of adsorbent. The chromatography requires comparatively long

time and the numerous evaporation in vacuum are not convenient in series analyses.

For our purpose, by the study of the transamination, the author has therefore tried to alter the present methods in such a way that they are suitable for series determinations of alanine, glutamic and aspartic acid, and the amounts of analysed substance are reduced as much as possible.

In the following pages a chromatographic method using aluminium oxide as adsorbent is outlined, which makes the determination of 5—30 micromols of the above-mentioned amino acids possible.

The amount of amino acid is computed from the nitrogen content of the eluate by the micro-Kjeldahl method.

Experimental.

First an attempt was made to carry out the analysis on a smaller scale by titration of the amino acids in alcohol. This gave satisfying results too, with regard to the accuracy, especially if thymolphthalein is used as indicator, and the endpoint of the titration is fixed by an indicator control. Also titration in acetone according to LINDERSTRØM-LANG (1927) gave good results in the case of neutral amino acids.

On the other hand it was impossible to titrate the aminodicarbonic acids because some of the aluminium oxide is dissolved by treating with potassium hydroxide solution, which disturbs the titration. This is not in accordance with the statement of WIELAND (1942) who does not, however, present experimental data. For that reason and because the evaporation in vacuum require much time and attention, the author tried to determine the amino acids in the eluates by the micro-Kjeldahl method. A condition is, that the reagents particularly the adsorbent, only contain a negligible amount of nitrogen. This is the case of aluminium oxide puriss. (MERCK). The content of soluble nitrogen is determined in the following way. 2 g Al_2O_3 previously treated as described later are eluted with 50 ml distilled water and then with 50 ml 0.05 N potassium hydroxide. The eluates are collected separately in 100 ml destruction flasks. The nitrogen content is determined as described under the analytical procedure. Simultaneously the nitrogen in 50 ml water and 50 ml 0.05 N potassium hydroxide are determined. They serve as blanks for respectively

aqueous and basic eluates. The analyses gave the values 0.002 mg water-soluble N and 0.000 mg alkali-soluble N in 2 g aluminium oxide. In order to obtain blanks as small as possible it is necessary to employ the purest analytical reagents, if convenient for very exact determinations solution in NH₃-free water.

The adsorptive power of the preparation is determined as follows. A series of adsorption columns of 2 g aluminum oxide are prepared. To these are added varying amounts of a glutamic acid solution containing 2 mg per ml. After rinsing with 10 ml distilled water the glutamic acid adsorbed is determined. It was found that up to 4.4 mg ~ 30 micromols glutamic acid are completely recovered. Amounts above 30 micromols are only incompletely recovered.

Apparature.

A glass tube 450 mm long and inside diameter of 7—8 mm is used as adsorption tube. In a distance of 100 mm from the bottom it is provided with a constriction, which is easily made in a sharply pointed flame. The tube is placed in the rubber-stopper of a cylindric glass, which is large enough to contain a receiver of 70 ml. Several columns are connected to the suction pump by a system of stop-cocks, as shown in the figure.

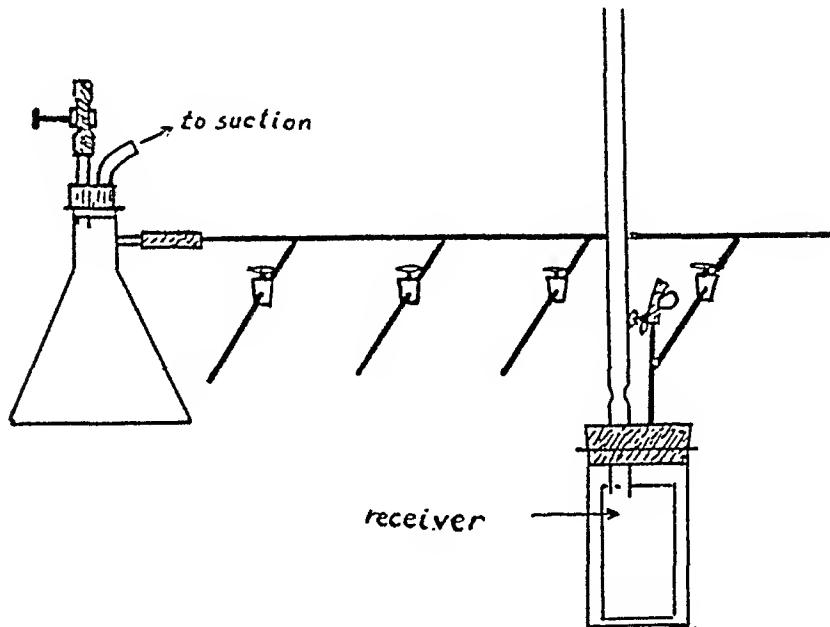


Fig. 1.

Analytical Procedure.

A. Separation of Glutamic Acid and Aspartic Acid from the Neutral Amino Acids.

Preparation of Adsorption Column. 2 g Al_2O_3 are shaken 5 min. with 6 ml N HCl. When the aluminium oxide has settled the supernatant fluid is removed by suction. Then the precipitate is shaken repeatedly with water until the water reacts neutral (litmus). The aluminium oxide, so treated, is washed with water into the adsorption tube, which is provided with a cotton plug at the constriction. When the aluminium oxide has settled the fluid is sucked off until only a few mm water are remaining above the column.

Adsorption of Amino Acids. The analysis, or perhaps the filtrate from a trichloroacetic acid precipitation, is pipetted with a semi-automatic pipette, LEVY (1938), into a small test tube. One drop of phenolphthalein is added and KOH-solution until a slightly pink colour appears. The analysis which is to contain 0.5—4 mg aminodicarboxylic acid, is transferred quantitatively into the adsorption tube by means of a Pasteur-pipette, glass and pipette being rinsed a few times with small portions of water. The analysis is now sucked through with a rate of one drop per 2 sec. and collected in the receiver (a vacuum of about 30—40 mm Hg is suitable). The column is rinsed with 15 ml water. This fraction contains the neutral and basic amino acids.

Eluation of the Aminodicarboxylic Acids. The receiver is now changed and the eluation with 3 ml 3 N KOH + 20 ml 0.05 N KOH is undertaken and the column is sucked dry. This fraction contains the aminodicarboxylic acids. A blank on pure water is run simultaneously in exactly the same manner.

Nitrogen Determination. The fractions are transferred quantitatively into a 100 ml destruction flask. The solution is acidified with diluted sulfuric acid and then 1 ml conc. H_2SO_4 , containing selenium as catalyst, is added. The water is evaporated over a small flame and the destruction is continued as usual; ammonia is distilled off in a micro-Kjeldahl apparatus. In the receiver are pipetted 10.00 ml 0.0100 N HCl. The excess of hydrochloric acid is determined iodometrically with 0.05 N sodium thiosulfate. A micro burette of 3 ml, graduated in 0.01 ml is used.

B. Separation of Glutamic and Aspartic Acid.

An adsorption column of 5 g Al₂O₃ is prepared in analogy with the procedure previously described. The activation is, however, performed with 15 ml N HCl. The neutralized analysis containing not more than 10 mg dicarbonic acid is transferred quantitatively to the column. Then is rinsed with 30 ml water. In the wash fluid the neutral amino acids are determined. Glutamic acid is eluted with 55 ml 0.5 N acetic + 10 ml H₂O, and the aspartic acid with 5 ml 3 N KOH + 40 ml 0.05 N KOH. The column is then sucked dry. Nitrogen determination is carried out as described above.

In the table the results of some analyses on mixtures of amino acids are given.

Table 1.

The determinations are carried out on 1.045 ml solution.

mg Amino acid per 1.045 ml mixture	Glutamic acid found mg	Δ	Δ in %	Alanine found mg	Δ	Δ in %
4.26 mg gl. ac.	4.29	+ 0.03	+ 0.7	—	—	—
	4.20	- 0.06	- 1.4	—	—	—
2.13 mg gl. ac.	2.12	- 0.01	- 0.5	—	—	—
	2.08	- 0.04	- 1.9	—	—	—
0.702 mg gl. ac.	0.710	+ 0.008	+ 1.1	—	—	—
	0.674	- 0.028	- 4.0	—	—	—
2.27 mg gl. ac.	2.30	+ 0.03	+ 1.3	4.53	- 0.03	- 0.7
4.56 mg al.	2.25	- 0.02	- 0.9	4.48	- 0.08	- 1.8
	Glutamic acid			Aspartic acid		
3.82 mg gl. ac.	3.89	+ 0.07	+ 1.8	3.07	- 0.03	- 1.0
3.10 mg as. ac.	3.73	- 0.09	- 2.1	3.15	+ 0.05	+ 1.6
3.82 mg gl. ac.	3.80	- 0.02	- 0.5	1.50	- 0.05	- 3.2
1.55 mg as. ac.	3.75	- 0.07	+ 1.8	1.57	+ 0.02	+ 1.3
1.83 mg gl. ac.	1.83	± 0.00	+ 0.0	2.10	- 0.04	- 1.9
2.14 mg as. ac.	1.86	+ 0.03	+ 1.6	2.13	+ 0.01	- 0.4

In order to obtain the greatest possible accuracy it is necessary to observe the amounts of elution fluids as closely as possible to eliminate the errors arising from the nitrogen content of the reagents, and carry through a uniform performance of the separate determinations. It is of great importance that the column never should be sucked dry between the different operations. Provided the determinations are carried out on deproteinized solutions,

sodium tungstate and sulfuric acid must not be used for the precipitation of proteins, as this involves an increased adsorption of the acid amino acids, whereby only a very incomplete eluation is obtained with the amounts of fluid employed. The accuracy of the method by careful performance is only depending on the accuracy of the micro-Kjeldahl method.

Summary.

On the basis of previously reported works by TURBA and WIELAND a chromatographic method for the quantitative separation and determination of glutamic acid and aspartic acid in a mixture of amino acids has been outlined. The method allows the determination of 5—30 micromols amino acids with the same accuracy as a micro-Kjeldahl determination. The method is especially apt for series analyses.

This work has been aided by a grant from the "Kong Christian X's Fond".

References.

TURBA, F., Ber. dtsch. chem. Ges. 1941. 74. 1829.
TURBA, F. and M. RICHTER, Ibidem 1942. 75. 340.
WIELAND, T., Hoppe-Seyl. Z. 1942. 273. 24.
WIELAND, T. and L. WIRTH, Ber. dtsch. chem. Ges. 1943. 76. 823.

From the Department of Physiology, University of Lund

The Presence of a Gastric Secretory Excitant in the Human Gastric and Duodenal Mucosa.

By

BÖRJE UVNÄS.

Received 9 June 1945.

From experiments on animals it is supposed that the HCl-secretion of the human stomach is initiated and maintained, at least, partially by the co-operation of a pyloric hormonal mechanism. However, deductive experimental evidence in favour of this view has scarcely been presented. A few mainly older clinical observations indicate that after a test meal the gastric secretion is abolished or considerably reduced on resection of the pyloric region (SCHUR and PLASCHKES 1915, LORENZ and SCHUR 1922, KLEIN 1927). Later investigators, among others WANGENSTEEN and co-workers (1940) deny that the secretory behaviour of the human stomach changes fundamentally after removing the pyloric region.

AMMON and LIM (1923), using a "secretin" extraction method and the method of DALE and LAIDLAW (1912) for preparing "gastrin", found either little or no "gastrin" in the human post-mortem pyloric mucosa. IVY and OBERHELMAN (1923) used the method outlined by KEETON and KOCH (1915) for preparing "gastrin". Given subcutaneously to Pavlov-pouch dogs their preparations from human post-mortem pyloric and duodenal mucosa stimulated the HCl-glands.

KOMAROV (1938, 1942) and UVNÄS (1942, 1943) reported the existence of a protein-like secretory excitant in the pyloric mucosa of cats, dogs and pigs. The agent was assumed to be identical with the gastric hormone, the existence of which was first supposed by EDKINS 1906. Since an investigation of the distribution of this

secretory principle in the human stomach might throw light on the hormonal control of gastric secretion, we have examined the stomach mucosa of some surgical cases for its content of the secretory agent. In addition we investigated the secretory activity of some preparations from human post-mortem gastric and duodenal mucosa.

Experimental.

The resected portions of stomachs from recently operated patients were kept in the ice box and transferred to the laboratory within 1—2 hours. The post-mortem material was usually obtained within 10—36 hours of death. The gastric mucosa was artificially divided into three portions — the pyloric, the boundary and the corpus region. The pyloric region comprised the mucosa to within about 4—5 cm from the pylorus, the corpus region the mucosa above the angular incisura except the distal half of the lesser curvature, and the boundary zone the mucosa between the pyloric and the corpus regions.

The active principle was extracted according to a modification of the method devised by MUNCH-PETERSEN, RÖNNOW and UVNÄS (1944). The mucosa was scraped off and boiled for 20 minutes in 10 volumes of N/10 HCl. Next day the material was filtered through gauze. N NaOH was added to the acid filtrate, to a pH of 3—4. Precipitated inactive material was removed by centrifuging. Inactive material was further precipitated by adding N NaOH to a pH of about 8. After centrifuging, the solution was acidified with N HCl to a pH of 3—4 and the active material precipitated with the same volume of 20 per cent trichloroacetic acid or 5 ml 5 per cent tannic acid solution. The active material was repeatedly washed in acetone and ether and dried in the air.

The secretory activity of the preparations was tested by slow intravenous injection for 30 minutes on anaesthetised cats as devised by UVNÄS 1945, the secretory response during 60 minutes after the beginning of the injection being taken as a measure of the activity of the preparations.

Results.

As seen in table 1 the pyloric mucosa from 4 cases of duodenal, 5 cases of gastric and 1 case of malignant gastric ulcer was examined for its content of a gastric secretory agent. All preparations except one caused a marked gastric secretion. In 2 cases of gastric carcinoma the one preparation was active, the other inactive. On the other hand practically no activity was observed in the preparations of the corpus mucosa. In only 2 out of 10 cases was an insignificant secretion obtained. The other preparations were quite inactive. The mucosa of the boundary zone holds an inter-

Table 1.

No.	Case	Age	Sex	Mucosal region						Final precipitant	
				Pyloric mucosa		Boundary-zone mucosa		Corpus mucosa			
				Mucosa (wet) g	Secretion ml	Mucosa (wet) g	Secretion ml	Mucosa (wet) g	Secretion ml		
M4	Duodenal ulcer	33	M	12	6.8	—	—	11	1.4	tr. ac.	
M6	" "	36	F	10	16.1	21.5	1.2	10	—	tr. ac.	
M7	" "	55	F	12	4.0	9	2.1	13.5	0	tr. ac.	
M9	" "	25	M	7	17.0	4	5.1	8	0	tr. ac.	
M8	Gastric	55	M	10	6.5	—	—	19	0	tr. ac.	
M23	" "	52	M	2	1.8	2	3.3	2	0	ta. ac.	
M26	" "	35	F	3	10.2	—	—	3	0	ta. ac.	
M27	" "	23	M	3	3.0	—	—	3	1.3	ta. ac.	
M29	" "	45	M	—	—	3	9.2	3	0	ta. ac.	
M18	Malignant gastric ulcer	46	M	3	11.2	3	0	3	0	ta. ac.	
M19	Gastric carcinoma . . .	75	M	3	3.0	6	0	—	—	ta. ac.	
M10	" "	52	M	12	0	8	0	—	—	tr. ac.	

Tr. ac. = trichloracetic acid. ta. ac. = tannic acid.

mediary position. Out of 8 preparations 3 were inactive, 2 evoked an insignificant secretion and 3 a distinct gastric secretion.

The post-mortem pyloric mucosa from 24 patients suffering from different diseases was examined. 6 preparations were inactive and 4 slightly active, while the remaining 14 evoked a marked gastric secretion. Due to the post-mortem changes of the corpus mucosa, this section was generally not examined. 5 preparations produced from the corpus were all inactive, as were also 4 preparations from the boundary zone mucosa.

Out of 15 duodenal preparations 9 were inactive, 3 showed an insignificant activity, and only 3 were slightly but distinctly active.

Discussion.

The investigated surgical material is rather small, but it definitely shows that the human gastric mucosa contains a gastric

secretory principle obtainable in the way outlined by MUNCH-PETERSEN, RÖNNOW and UVNÄS for isolating a secretory agent from the pyloric mucosa of cats, dogs and pigs. Our observations are too few to permit definite conclusions about the distribution of the secretory principle, but they strongly indicate that the principle is chiefly if not entirely localized into the pyloric mucosa.

The secretory principle isolated by us is not identical with the "gastrin" obtained from post-mortem pyloric and duodenal mucosa by AMMON and LIM or Ivy and OBERHELMAN. As pointed out by these authors as well as by DALE and LAIDLAW and KOCH, LUCKHARDT and KEETON (1920), the "gastrin" and "gastrin bodies" extracted by their methods are histamine or some related imidazol derivates. The secretory principle extracted by us is a protein-like substance.

According to MIYAGAVA (1921) and DEBEYRE (1924), the "pyloric" glands in man occupy an area corresponding to 4.5—5.5 cm on the lesser curvature and 4.0 cm on the greater curvature. We have not intended to co-ordinate the histological structure or the mucosa with the presence of the secretory principle, but our experience is not contradictory to the opinion that the secretory principle is localized in the mucosa containing "pyloric" glands.

As discussed in previous papers from our laboratory, the pyloric secretory principle is probably identical with the gastric hormone, gastrin. This being the case, it will be desirable to resume the clinical investigations of the human gastric secretory mechanism. As mentioned above, later investigators in this field usually claim that the secretory behaviour of the human stomach is not altered after resection of the pyloric region. As far as we can see most of these experiments are inconclusive, however. Many workers (e. g. WANGENSTEEN and co-workers) use alcohol and histamine as stimulating agents, both of which stimulate the HCl-glands directly without involving any pyloric hormonal mechanism. Usually only the acidity of the gastric secretion was determined, and never was the possible rôle of the intestinal phase taken into consideration.

As clinical gastric analysis was performed only in a few of our cases, any quantitative correlation between the mucosal content of the secretory principle and the secretory function of the stomachs must be omitted. This problem will be dealt with in later experiments.

Of great interest is the presence of a secretory agent in the duodenal mucosa. It is true that out of 15 preparations 9 were

quite inactive and only 3 significantly active. But owing to the fact that the secretory agent is rapidly destroyed by trypsin, it is rather surprising that the agent does not quite disappear soon after death. It has been discussed whether the intestinal phase of gastric secretion is mediated in a humoral or a hormonal way. KOMAROV reports that a gastric secretory principle can be obtained from the duodenal mucosa of pigs. We have not been able to conform this result. The presence of gastrin in the duodenal mucosa of man might indicate that the intestinal phase is mediated in a hormonal way.

Summary.

A protein-like gastric secretory principle is found in the human pyloric mucosa.

References.

AMMON, S. E., and R. K. S. LIM, Brit. J. Exp. Path. 1923. 4. 27.
 DALE, H. H., and P. P. LAIDLAW, J. Physiol. 1910. 41. 318.
 DEBREYRE, A., Arch. d'Anatomy 1924. 3. 37.
 EDKINS, J. S., J. Physiol. 1906. 34. 133.
 IVY, A. C., and H. A. OBERHELMAN, Amer. J. Physiol. 1923—24. 67.
 450.
 KEETON, R. W., and F. C. KOCH, Ibidem 1915. 37. 481.
 KLEIN, E., J. Amer. med. Ass. 1927. 69. 1235.
 KOCH, F. C., A. B. LUCKHARDT, and R. W. KEETON, Amer. J. Physiol 1920. 52. 508.
 KOMAROV, S. A., Ibidem. 1938. 36. 514.
 KOMAROV, S. A., Rev. Canad. Biol. 1942. 1. 191.
 LORENZ, H., and H. SCHUR, Arch. klin. Chir. 1922. 119. 239.
 MIYAGAVA, Y., J. Anat., Lond. 1921. 55. 56.
 MUNCH-PETERSEN, J., G. RÖNNOW, and B. UVNÄS, Acta Physiol. Scand. 1944. 7. 289.
 SCHUR, H., and S. PLASCHKES, Mitt. Grenzgeb. Med. Chir., 1915. 26. 795.
 WANGENSTEEEN, O. H., R. L. VARCO, L. HAY, S. WALPOLE, and B. TRACH, Ann. Surg. 1940. 112. 626.
 UVNÄS, B., Acta Physiol. Scand. 1942. 2. suppl. XIII. Diss.
 UVNÄS, B., Ibidem 1943. 6. 97.
 UVNÄS, B., Ibidem 1945. In press.

An Experimental Study of Disorders in the Permeability of the Cerebral Vessels ("the Blood-Brain-Barrier") Produced by Chemical and Physico-chemical Agents.

By

TORE BROMAN and A. M. LINDBERG-BROMAN.¹

Received 13 June 1945.

Introduction.

In vital-staining experiments GOLDMANN (1913) observed that trypan blue (trpb), which quickly makes its way from the blood into the tissues, is unable to penetrate into the CNS² by this route, whereas, injected into the cerebrospinal fluid, the dye passes without difficulty into the brain substance. GOLDMANN'S observations have later been verified by several investigators and have been proved to be valid also for other substances (dyes, drugs, toxins and others). Indeed, the existence of a special "mechanism" regulating the exchange of substances between blood and brain has been generally accepted, but opinions still differ widely as to its nature and its localization. Investigations carried out by SPATZ (1924, 1933), FRIEDEMANN and ELKELES (1932, 1934), BROMAN (1940) and others seem to prove that the inhibitory effect is localized to the walls of the cerebral vessels and that it is connected with the permeability of these vessels. This view has lately received further support by investigations of the passage of different ions (WALLACE a. BRODIE 1937, KROGH 1945). According to the conception of the BBB here outlined, vital-staining of the CNS by means of trpb (or other

¹ The authors of the present study were enabled to perform their investigations at the Physiological Institutes of Lund and Stockholm by the courtesy of Professors G. KAHLSON and U. v. EULER respectively.

² Central Nervous System.

dyes of the same kind) indicates a disorder in the permeability of the cerebrospinal vessels.

Since trpb was shown not to pass the walls of the cerebral vessels during normal conditions, it seemed natural to use it as a "colour indicator" of cerebral vascular lesions. As far as we have found, MACKLIN and MACKLIN (1920) were the first to adopt this method in their study of mechanical lesions of the CNS, followed later on by MENDEL (1928), BELLAVITIS (1929), RISER (1929), RUSSEL (1929), SCHMID (1931) and others, who also investigated thermal lesions. In these cases it was mostly a question of gross injuries by external violence and staining of the brain might follow as a result of the bleeding. Thus, this cannot without further analysis be interpreted as depending solely on increased vascular permeability.

In cerebral lesions caused by blood-borne agents other investigators have been successful in demonstrating disorders of the permeability by means of the colour indicator method. Thus SKOOG (1937) found in experiments on guinea-pigs with a sheep-hemolytic rabbit serum a vital-staining of the damaged area. As far as we have found, his publication constitutes the only investigation hitherto published concerning injuries to the BBB directly produced by a chemical agent and analyzed by the colour indicator method. By the same method BROMAN (1938, 1940) was able to demonstrate an increased permeability in micro-embolic lesions of the cerebral vessels.

All the investigators mentioned above have used essentially the same procedure in their experiments, viz.:

1. A limited part of the CNS is injured.
2. Dye is administered parenterally.
3. After a suitable interval the animal is killed and its brain examined. Disorders of the barrier-function can then be observed through the staining of the damaged area, while other parts of the brain remain unstained and serve as a control that no source of errors has crept in unnoticed.

Own Investigations.

General disposition of the experiments.

Our investigation is designed to be only a rough survey of the influence of some chemical and physico-chemical agents on the BBB. The agents (see pages 105—106) are rather arbitrarily chosen, and

the investigation does of course not aim at being complete. Stress has been laid mainly upon methodological questions. It must be emphasized that the results obtained have bearing only upon the so-called *barrier*-function of the cerebral vessels, since it is possible to demonstrate by the colour indicator method only such disorders in the cerebral vascular permeability as involve an injury to the BBB. Therefore, our investigation gives no first-hand information regarding the ability of the substances to penetrate into the CNS, nor about their effect on the nerve cells.

We have used guinea-pigs, rabbits and cats, as is seen from the tables or described below. Regarding all these species earlier investigations have shown that trpb never penetrates into the CNS unless there is an injury to the BBB. In every case the barrier-function was tested by means of trpb according to the colour indicator method elaborated by BROMAN (1938, 1941) or some modification of it. — After the different ways in which the agents were administered the experiments have been arranged into three series:

- I. Injection into the carotid artery.
- II. Local application on the pial vessels.
- III. Perfusion of the brain hemisphere.

In the first series the agents were injected into the carotid artery according to principles laid down by FORSSMAN (1927) and by FRIEDEMANN and ELKELES (1931). The injected fluid mixes with the blood stream and follows it to the brain. After administration of trpb the animals were killed and the brains examined post mortem. — This procedure is technically simple and in most instances quite satisfactory as a routine method of examining experimental injuries to the BBB. When, however, the effect of chemical and physico-chemical agents is to be tested, it is evident that serious objections may be raised against this method, as the dilution with blood is unknown and varies from case to case, and, consequently, the actual concentration of the noxious substance cannot be obtained.

In the second series we tried to escape this inconvenience by local application of the agents on the pial vessels, made accessible through a trephine opening. As shown by BROMAN (1941) these behave with regard to their permeability properties in the same manner as the intracerebral vessels. A possible passage of dye was controlled by intravital observations, and the minimum concentrations, at which a damage of the BBB appeared were thus es-

tablished. — From a technical point of view the arrangements present some difficulties, especially in small animals, which is why we mostly used rabbits and cats in these experiments. The method is, however, open to some criticism regarding the application of the agents. It is generally accepted that the permeability of the vessels is linked to the *endothelial* layer, and there are, as mentioned above, good reasons to suppose that such is also the case with the barrier-function. If so, the locally applied agent reaches the barrier membrane only after permeation through the adventitia and media; during this time it may still be diluted to an unknown degree (the degree of dilution being dependent on the rate of penetration); perhaps it may also be neutralized or counteracted by substances diffusing from the blood in the opposite direction. Thus the validity of the results may be more or less restricted.

In order to bring a solution of known concentration into direct contact with the vascular endothelium we worked out another modification of the method and used it in the third series of experiments. The brain hemisphere was perfused for about a minute through the carotid artery at a pressure high enough to replace the blood by the test-solution. During the perfusion the pial vessels were observed directly in order to control that the blood had been effectively replaced by the perfusion fluid, and also afterwards to observe a possible passage of trpb out of the vessels. — The main objection against this method is that the time of perfusion with toxic substances must not exceed a few minutes, if the animal is to survive. Probably an injury to the BBB could be brought about at lower concentrations, if the time of action could be prolonged as it is probable that several agents penetrate rather slowly into the walls of the cerebral vessels. There is also a possibility that different substances may diffuse from the brain into the vessels and counteract the effect of the agents. Perhaps it must also be taken into account that the noxious effect of certain agents in some way depends on the presence of elements which normally occur in the blood, as will be discussed below in connection with the experiments concerning allergic reactions.

The agents examined.

1. Hypo- and hypertonic salt solutions. (Changes of osmotic pressure.)
2. Solutions of varying H-ion concentration.

3. Bile salts: sodium-glycocholate, sodium-desoxycholate.
4. Ethyl alcohol.
5. Toxins: a) bacterial: diphtheria,¹ tetanus¹ and staphylococcus² toxins;
b) animal: cobra³ and bee venoms.
6. Substances acting on the vascular walls:
adrenaline, acetylcholine,
histamine, Witte peptone.
7. Substances with allergic effect:
a) antigens: horse serum, ovalbumin;
b) FORSSMAN-antibodies: sheep-hemolytic rabbit serum⁴
(in guinea-pigs).

ad (3) Bile salts were examined because of their known ability of increasing the permeability of membranes.

ad (5). FRIEDEMANN and ELKELES (1934) have shown that diphtheria, tetanus and probably also staphylococcus toxins cannot pass from the blood into the CNS, while cobra venom easily does so. We thought it of interest to supplement their statements with an analysis of the influence which these toxins may exert on the BBB.

ad (6). Adrenaline was submitted to renewed examination as one of us has found in earlier experiments on guinea-pigs that it sometimes injured the BBB (BROMAN 1940). — Histamine is known to increase strongly the capillary permeability in other parts of the body, rendering the capillary wall permeable to plasma proteins. For this reason it seemed probable that histamine would injure the barrier-function too. — Witte peptone resembles in many respects histamine in its poisonous effect upon capillaries. "Peptone shock" may give almost the same symptoms as histamine shock (and anaphylactic shock).

ad (7). *A priori* it also seems plausible that allergic reactions may alter the function of the BBB, as an increase of the capillary permeability is an outstanding characteristic of allergic conditions in other organs. Experimental evidence concerning this matter has been furnished by SKOOG (1937) in his investigations of cerebral symptoms in guinea-pigs induced by "reverse anaphylaxis" (FORSSMAN's carotid syndrome).

¹ Kindly put to our disposal by Statens Bakt. Lab.

² » » » » » » prof. FORSSMAN.

³ » » » » » » HAMMARSTEN.

⁴ » » » » » » » LINDAU and doc. G. HAMMARSTEN.

The first series of experiments.

Mostly guinea-pigs were used, and only a few experiments were, for special purposes, performed on other animals (rabbits, cats, dogs). Solutions of varying H-ion concentration were not tested with this method.

In *guinea-pigs* the injection of the noxious agents was performed according to FORSSMAN (1926) and SKOOG (1937). Under local anaesthesia or narcosis (ether or urethane) the right carotid artery was tied distally. 0.5—1.0 ml. of the test-solution was injected during 15—25 sec. into the artery in centripetal direction (*i.e.* into the proximal part of the artery). Thus injected, the main part of the fluid will follow the blood stream through the right subclavian artery and part of it pass through the right vertebral artery to the brain stem. When the injection is given under local anaesthesia the animal may be released afterwards and examined for possible cerebral symptoms (FORSSMAN's carotid syndrome). The dose of the tested substances was increased until damage of the vascular permeability function was attained or general toxic symptoms made the use of higher concentrations impossible. A saturated solution of trpb in saline was then administered intravenously under narcosis in an amount of 10—20 ml. Artificial respiration was resorted to in cases of impending respiratory failure — a fairly common complication with guinea-pigs, even if the dye is given very slowly. After an interval of at least 10 min. from the beginning of the infusion of the dye the animal was killed by bleeding (the chest was opened and the heart cut up), and the content of the cerebral vessels thoroughly washed out by immediate perfusion with saline solution through a cannula inserted into the thoracic aorta, followed by a 20 per cent formaldehyde-solution in order to get the brain fixed *in situ*. The perfusions were performed at a pressure of about 100 cm. of water, the amounts of fluid being 100 ml. respectively. The brain was then removed and examined macroscopically. The result is considered positive when the region supplied by the right vertebral artery is stained. Sometimes frozen sections were made for microscopic control.

In *other animals* the injection was made with a fine cannula directly into the blood stream in the non-ligated carotid artery. By this route the agent reaches the cerebral hemisphere. The amounts of trpb and of perfusion fluids were increased in relation to the body weights of the animals.

Results.

Table 1 a.

Animal	Agents	Conc. %	Cerebral symptoms	Staining	Notes
1, 2	Aq. dest.	—	0	0	inj. of 10 ml/2 min.
3	Sodium chloride	10	?	0	
4	" "	20	+	+	
5	Sod. glycocholate	2	0	0	
6	" "	10	+	++	cerebral edema a. stasis
7	Sod. desoxycholate	0.5	(narcosis)	+	" " "
8	" "	2	+	++	" " "
9	Ethyl alcohol ..	50	0	0	
10	" " ..	70	?	+	uncertain cerebral edema ..
11	" " ..	96	+	++	" " ..
12, 13	Diphtheria toxin		0	0	
14, 15	Tetanus toxin ..		0	0	
16	" " ..	0.001 ml s.c.		0	tetanus after 2 days; inspection also of spin. cord
17-19	Staphylococcus toxin		0?	0	
20	Cobra venom ..	0.07	(narcosis)	++	no cerebral edema or stasis
21	" " ..	0.1	+	++	" "
22	Bee venom	0.1	0	0	
23, 24	" "	0.1	+	+	cerebral edema a. stasis
25, 26	Adrenalin	0.02	(narcosis)	+	dotted staining of whole CNS
27	"	0.02	"	0	
28-30	Acetylcholine ..	0.1-5	0	0	
31	Histamine	0.005	(narcosis)	0	ether-narcosis before inj.
32	"	0.05	"	0	urethane-narcosis before inj.
33, 34	"	0.01	"	0	ether-narcosis after inj.
35, 36	"	0.005-0.05	0	0	no narcosis (symptoms of shock in all cases)
37, 38	Witte peptone .	5	0	0	symptoms of shock

Table 1 b.

Animal	Species	Agents	Conc. %	Narcosis	Staining	Notes
1-3	Rabbit	Adrenalin	0.1-0.2	Urethane	0	2-5 injections
4, 5	Cat	"	0.1-0.2	Chloralose	0	2 " "
6-8	"	Histamine	0.1-10	Chloralose	0	2-4 " . symptoms of shock

Animal	Species	Agents	Conc. %	Narcosis	Staining	Notes
9, 10	Cat	Histamine	1—20	Ether	0	2—4 injections, symptoms of shock
11, 12	Dog	*	2—5	Chloralose	0	2—3 injections, symptoms of shock

The second series of experiments.

These experiments were as a rule performed on rabbits and cats, guinea-pigs being less suitable on account of the technical difficulties of the method.

Under narcosis (ether, urethane) the skull was trephined on one side and the dura removed. Great care was taken not to injure the pial vessels; the surface was at intervals moistened with Ringer's solution. Through a cannula inserted into the femoral vein trpb was infused continuously during the experiment (lasting $\frac{1}{2}$ —1 hour), the total amount of liquid varying from 75—200 ml. in animals weighing 1,500—3,000 gm.

The test-solution was brought into contact with the pial vessels either by dropping or by means of a cotton-wool pellet soaked with the liquid and cautiously placed on the pial surface. The agent was usually allowed to act for one minute, but in some cases the contact was prolonged to 10 minutes. Afterwards the brain surface was carefully inspected for some minutes through a microscope at a magnification of about $50 \times$; in most cases, however, a magnification of about $10 \times$ proved to be quite sufficient and could be obtained by an ordinary lens.

A positive result is indicated when the tissue around the vessels is stained; scattered cells with deep blue colour are often seen against a more diffusely stained background. Sometimes the dye is actually seen to "explode" out of the vessels within a few seconds; if the damage is less pronounced on the other hand, one minute or two may elapse before a slight staining can be noticed.

— Post mortem examination was usually not undertaken.

There is often enough a tendency to cerebral edema in these experiments and attention must be paid so that the brain does not become incarcerated in the trephine opening with an ensuing obstruction of the veins, as the BBB-function would then be seriously impaired. This is most easily avoided by making the trephine opening as large as possible. — The window technique

does not offer any great advantages in this respect and is furthermore unnecessary, as no determination of the vascular calibre is intended; the air has no marked influence upon the barrier-function and pressure conditions are of no importance as long as there is no stasis. Without a window there is also a possibility of getting the injury localized to only a part of the free surface, the other part being used for comparison in doubtful cases. This is of some value for determining the minimum concentration with injurious effect.

Results.

Table 2.

Animal	Species	Agents	Concen- tration	Stain- ing	Notes
1—5	Rabbit	Aq. dest.	—	0	time of applic. 1— 10 min.
6—8	Cat	” ”	—	0	” ”
9—11	Rabbit	Sodium chloride	3%, 5% 10%, 20%	0 +	
12	”	” ”	5%, 6%, 7%	0	
		” ”	8%, 9%, 10%	+	
13, 14	Cat	” ”	10% 15%	0	
15	Rabbit	Citrate/HCl-buffer sol.	pH 3, 2, 1	0	
16	”	Lactic acid 1% in aq. dest.	pH 1	0	
		Acetic acid 1% in aq. dest.		0	
17, 18	Cat	Citrate/HCl-buffer sol. Lactic acid 1% in aq. dest.	pH 1	0	
		Acetic acid 1% in aq. dest.		0	
19	Rabbit	Borate/NaCl-buffer sol.	pH 8, 9	0	
20	”	” ”	pH 10	+	
		NaHCO ₃ 1%	pH about 8	0	osm. pr.=0.75% NaCl
		9/10 NaHCO ₃ 1% } 1/10 Na ₂ CO ₃ 1% }	pH ” 9	0	” ”
		Na ₂ CO ₃ 1%	pH ” 10	+	” ”
21—23	Cat	Borate/NaOH-buffer sol.	pH 8, 9	0	
		” ”	pH 10	?	
		” ”	pH 11	+	
24, 25	Rabbit	Sod. glycocholate	1 % _{oo}	0	
		” ”	5% _{oo} , 10% _{oo}	+	
26, 27	Cat	” ”	1 % _{oo}	0	
		” ”	5 % _{oo}	+	

Animal	Species	Agents	Concen-tration	Stain-ing	Notes
28	Rabbit	Sod. desoxycholate . . .	1 % _{oo} 5 % _{oo} 1 % _{oo} 2 % _{oo} 3 % _{oo} , 5 % _{oo}	0 + 0 (+) +	
29, 30	*	*			
31	Cat	*	5 % _{oo} 10 % _{oo}	0 +	
32, 33	Rabbit	Ethyl alcohol	10%, 25%, 50%	0	
34, 35	*	*	10%, 25%, 30% 40%, 50%, 75%	0 +	
36—38	Cat	*	20%, 25%, 30% 40% 50%	0 (+) +	
39, 40	Rabbit	Diphtheria toxin		0	
41, 42	*	Tetanus toxin		0	
43, 44	*	Staphylococcus toxin		0	
45—47	*	Cobra venom	1 % _{oo} 1% 5% 1 % _{oo} , 2 % _{oo} , 6 % _{oo} 1 % _{oo}	0 (+) +	
48	*	*		0	
49, 50	*	Bee venom	1 % _{oo}	0	higher conc. not available
51—53	*	Adrenaline	1 % _{oo} , 1 % _{oo} , subst.	0	
54	Cat	*		0	
55	Guinea pig	*	subst. 1 %	0	
56, 57	Rabbit	Acetylcholine	1 % _{oo} , 1 % _{oo}	0	
58, 59	*	*	10% in aq. dest. subst.	0	
60—63	*	Histamine	1%, 10%, subst.	+	diapedesis of red corpuscles
64—68	Cat	*	subst.	0	1—10 min.
69, 70	Rabbit	Witte peptone	substane	0	10 min.

The third series of experiments.

These experiments were mostly performed on rabbits (generally weighing $1\frac{1}{2}$ kg.), and only in a few instances on cats or guinea-pigs. A cannula was inserted into the trachea under narcosis (urethane, dial). The carotid artery was laid bare on one side and ligated. Another cannula was inserted into its distal (cranial) end. The skull was then trephined over the hemisphere of the same

side. The dura was left intact, if it was possible to observe the pial vessels through it (often possible in rabbits, seldom in cats).

The test-solutions were infused via the carotis-cannula at a pressure a little above the blood pressure by means of a reservoir which could be elevated. The pial vessels were observed during the perfusion which as a rule lasted for 1 minute and could be repeated several times, if the solution was not too injurious. In order to prevent the toxic substances from entering the general circulation, the blood from the jugular vein of the same side was allowed to run out during the perfusion. In case of respiratory failure artificial respiration was performed.

Immediately after the perfusion, when the animal's own blood was again flowing through the brain, 50 ml of trpb was infused intravenously for about 5 minutes. The brain surface was inspected as in series 2. When there was any passage of dye or if the animal died, the experiment was finished as in series 1 in order to make a post mortem examination possible, too.

A positive result, observed intravitaly, can thus be verified by post mortem examinations of brain sections. Usually one finds the whole hemisphere blue and sometimes also a part of or the whole other hemisphere and the brain stem (due to high perfusion pressure).

Substances which gave negative results in the previous series were not tested with this method. Exceptions were made for aqua destillata and acid solutions, as it could be suspected that the negative results were caused by methodological circumstances (dilution with blood etc.). Bee venom was not available in quantities big enough for any experiments in this series.

Results.

Table 3.

Animal	Agents	Concentration, time of application	Staining	Notes
1-3	Aqua destillata	1/2 min, 1 min	0	cerebral edema
4	" "	2 min	?	
	" "	3 min	+	cerebral edema, passage of dye only around intracerebral arteries
5-8	Sod. chloride	2%	0	
	" "	5%, 6%	+	scattered areas of staining
9	Phosphate-buffer sol.	pH 6	0	

Animal	Agents	Concen-tration, time of appliaction	Stain-ing	Notes
10	Citrate/NaOH-buffer sol.	pH 5.2	0	
	Citrate/HCl-buffer sl.	pH 4.5	0	
11	" "	pH 4.0	+	
	Acetic acid 1%	pH 3.2	++	
12	NaHCO ₃ 1%	pH about 8	0	osm.pr.=0.75% NaCl
13	9/10 NaHCO ₃ , 1% 1/10 Na ₂ CO ₃ , 1% }	pH > 9	0	" "
14	Na ₂ CO ₃ 1%	pH > 10	++	" "
15	Sod. glycocholate ..	0,1 %	0	
16	" "	1 %	++	
	" "	0.5 %	+	
17	Sod. desoxycholate ..	0.2 %	0	
18	" "	1 %	++	
	" "	0.4 %	?	
19, 20	Ethyl alcohol	1%, 10%	0	15% — sudden death
	" "	20%	?	sudden death
21	" "	1%, 5%, 10%	0	exp. on cat
22	" "	15%	++	" "
23	Cobra venom	0.1 %	0	
24—26	" "	0.3 %, 5 %	?	sudden death

Experiments with allergic lesions.

As mentioned in the introduction SKOOG has demonstrated a disturbance of the cerebral vascular permeability in experiments with reverse allergy. His experiments were performed in accordance with the method used in our first series. We have here renewed his experiments with special regard to the localization and duration of the disturbances. We have moreover added some experiments according to the methods in our series 2 and 3. Since experiments with reverse allergy can hardly be made on other laboratory animals than guinea-pigs, we have used only this species. — The results are shown in tables 4 a, b, c.

We have also tried to produce disturbances of the cerebral vascular permeability by the usual allergic reactions. After sensitizing the animals — guinea-pigs or rabbits — with horse serum or ovalbumin, we tried to provoke an allergic reaction in the brain by local application of the antigen on the pial vessels or injection into the cerebral arteries or into the brain substance. The results are shown in tables 5 a, b, c.

Results.

Table 4 a.

Animal No.	Cerebral symptoms	Time betw. inj. of serum a. dye	Staining	Localization	Bleedings
1.....	(narcosis)	2 min	+++	Ctempo	0
2.....	+	5 "	+++	"	0
3.....	++	10 "	+++	"	0
4.....	++	20 "	+++	"	0
5.....	+++	1 hour	+++	Ctempo	0
6.....	++	1½ "	+++	"	0
7.....	++	4 "	+	P	+
8.....	+	4 "	0	—	0
9.....	+++	7 "	+	po	+
10.....	++	11 "	0	—	0

Sheep-hemolytic rabbit serum injected into the carotid artery on guinea-pigs ad modum FORSSMAN-SKOOG.

(C = cerebrum, c = cerebellum, m = mesencephalon, p = pons, o = oblongata.)

Table 4 b.

Animal	Dilution of the serum	Staining
1, 2.....	1/1	0
3, 4.....	1/2 in heparinized guinea-pig's plasma	0
5.....	1/2 " " " blood	0
6.....	1/2 " guinea-pig's serum	0
7.....	1/2 " defibrinated guinea-pig's blood	0

Sheep-hemolytic rabbit serum applied locally on the pial vessels of guinea-pigs.

Table 4 c.

Animal	Amboceptor-concentration of the serum	Dilution of the serum	Staining	Localization
1-3	1 : 3000	1/15 — 1/5 in 0.9 % NaCl	0	—
4-6	1 : 3000	1/5 in heparinized guinea-pig's plasma	0	—
7	1 : 5000	1/5 " guinea-pig's serum	0	—
8	1 : 3000	1/5 " heparinized guinea-pig's blood	+	C
9	1 : 5000	1/5 " defibrinated " "	++	Cc
10, 11	1 : 3000	1/5 " suspension of guinea-pig's blood corpuscles in 0.9 % NaCl	+	C
	1 : 5000	1/5 corpuscles in 0.9 % NaCl	++	C

Sheep-hemolytic rabbit serum perfused through one hemisphere of guinea-pigs during $\frac{1}{2}$ —1 minute.

Table 5 a.

Animal	Sensitizing dose of antigen	Dose of antigen inj. in a. carot.	Cerebral symptoms	Anaphylactic shock symptoms	Staining
1—2 G	$h\frac{1}{4} + \frac{1}{2} + \frac{3}{4}$ ml s.e.	0.6—1 ml 10%	0	++	0
3—5 G	o 5, 15 or 50 mg i.p.	0.5—1.0 ml 5%	0	++	0
6—9 R	$h\frac{1}{2} + 1 + 1\frac{1}{2}$ ml s.e.	2 ml 20%	0	+	0
10, 11 R	h 1 ml i.c.	2 ml 25%	0	0	0

Injection of horse serum (h) or ovalbumin (o) into the carotid artery (as in the first experimental series) after sensitization of the animals — guinea-pigs (G) and rabbits (R) — by subcutaneous (s.c.), intraperitoneal (i.p.) or intracisternal (i.c.) injections 3—6 weeks before.

Table 5 b.

Animal	Sensitizing dose of antigen	Concentration of the locally applied antigen	Staining
1 R	$h\frac{1}{2} + 1 + 1\frac{1}{2}$ ml s.e.	1/1	0
2 G	$h\frac{1}{4} + \frac{1}{2} + \frac{3}{4}$ *	1/1	0
3,4 R	h 0.5—1 ml i.e.	1/1	0
5,6 R	h 1+1 ml i.e.	1/1	0
7,8 R	o 20—100 mg i.e.	substance	0
9 R	h 0.5 ml intracerebrally	1/1 on the place of previous inj.	0
10 R	h 0.5+1 ml *	1/1 * * * * *	0
11 G	$h\frac{1}{4} + \frac{1}{2} + \frac{3}{4}$ ml *	1/1 * * * * *	0

The same antigens as in table 5 a applied locally on the pial vessels.

Table 5 c.

Animal	Sensitizing dose of antigen	Intracerebrally injected dose	Extensive zone of staining
1,2 R	2 ml intraven.	0.1 ml	0
3,4 R	2 *	0.1 *	+
5,6 G	$\frac{1}{2} + 1$ ml i. p.	0.1 *	+

Horse serum injected intracerebrally on previously sensitized animals. Injections of 0.1 ml 0.9% NaCl were made in the other hemisphere for comparison.

Discussion.

Our experiments have shown that the barrier-function of the cerebral vessels may be disturbed by several chemical and physico-chemical agents, but that it is remarkably resistant against others. A summary of our results is given below (table 6). As is seen from

this, a disturbance was most easily established by bile salts, ethyl alcohol, and venoms, while bacterial toxins, peptone, histamine, acetylcholine and adrenaline (with certain exceptions) were without effect on the barrier-function. Changes in the osmotic pressure and the H-ion concentration had an influence only if they were rather extreme. Allergic reactions caused an injury of the BBB only under certain conditions.

In the following our findings will be subjected to a closer discussion.

I. The lowest permeability-disturbing concentration of the tested substances. — Experiments with changes of the osmotic pressure and the H-ion concentration.

In the experiment series 2 and 3 attempts were made to estimate the minimum concentration of the substances able to disturb the cerebral vascular permeability. A comparison between the two series proves that the permeability is disturbed more easily and at lower concentrations from the intima-side than from the adventitia-side of the vessels. This observation agrees with our opinion that the BBB function is a special quality of the general permeability function in the cerebral vessels and that it is maintained by the endothelium.

The minimum concentration in question is, however, not a fixed quantity but must be put in relation to the time-factor, *i.e.* the time during which the agent has been allowed to exert its influence. For practical reasons this time had to be restricted, especially in series 3. Bearing this restricted time in mind, it is obvious that some other factors acquire a certain importance, too. Thus, the different penetration rate of various substances through the vascular endothelium may give rise to different equilibria of concentration between the blood and the endothelial cells in experiments of short or of long duration respectively. Furthermore the effect of some of the agents — especially aqua destillata, sodium chloride, acids and alkalis — might be counteracted by the diffusion in opposite direction of substances occurring in tissues and blood, and this influence must be more powerful in short-time experiments, before the body's resources are used up. It also seems natural that this diffusion should be more pronounced from the blood to the pial side of the vessels than in the other direction — a circumstance that at least partly could explain the fact that aq. dest. and acids had hardly any influence on the barrier-function when applied locally on the pial vessels.

It should, however, be emphasized that the diffusion of neutralizing substances is of importance mainly in the experiments where changes of the osmotic pressure and the H-ion concentration are involved and seems to be an unavoidable source of errors in these experiments. They may therefore seem to be of less value but are at all events of interest from a methodological point of view in demonstrating how much — or more properly, how little — the barrier-function is influenced by such alterations in experiments of the present type. These factors, therefore, can hardly come into account as sources of error in our experiments with other agents.

II. Experiments with bile salts and ethyl alcohol.

In all our series these substances elicited a disturbance of the barrier-function. There arises the question, if this effect may be of clinical importance. We think it possible that, in such diseases of the liver which give rise to high blood-concentrations of bile-salts, a barrier-injurious effect could appear. This might be an explanation of the correlation sometimes observed between symptoms from the liver and from the CNS.

The injurious concentrations of the alcoholic solutions in our experiments are, on the other hand, so high that it seems rather improbable that alcohol-intoxication in man could cause a damage of the BBB. The effect of ethyl alcohol on the CNS rather serves as an illustration of substances which damage the nerve cells in concentrations far below those which derange the barrier-function (cfr the analogous behaviour of hypotonic salt solution).

III. Experiments with toxins and venoms.

Earlier investigations by FRIEDEMANN and ELKELES (1934) have shown that the toxins of diphteria, tetanus and staphylococcus cannot penetrate into the CNS from the blood, while cobra venom does so. In our experiments we were able to demonstrate that the above-mentioned toxins do not have any injurious effect on the barrier-function, while cobra venom has such an effect. Of course this investigation is too incomplete to allow of any safe generalizations. It seems reasonable, however, to suggest that proteins must damage the vessels before they are able to pass into the cerebral tissue and that those which do not affect the cerebral vessels never penetrate their walls.

IV. Experiments with adrenaline.

In experiments with guinea-pigs BROMAN (1940) often found dotted staining throughout the whole CNS after intravenous

injections of trpb and adrenaline, but not after injections of trpb alone. This was explained as an injurious effect of trpb on the cerebral vessels — latent until the blood pressure was raised. In later experiments (BJERNER, BROMAN and SWENSSON 1943) it was found that a similar passage of dye could be caused in rats, too, after intravenous injections of trpb without adrenaline. On the other hand it was never seen in rabbits or cats. The present experiments confirm these earlier results.

That the passage of dye into the CNS in the above-mentioned experiments on guinea-pigs after injection of adrenaline does not indicate a direct effect of the adrenaline itself is proved by the negative results in series 2 and by the results in series 1, where the peculiar staining was found in the whole brain and not only on the side of injection.

It seemed strange that this kind of staining should occur only in small animals. In experiments with mechanical injuries to the cerebral vessels (not yet published) BROMAN has found that a disturbance of the barrier-function can be produced in different animals — including rabbits and cats — by raising the venous pressure. In a case of brain hernia, for instance, the passage of dye began already within one minute. In some experiments on guinea-pigs in which the venous pressure was raised by pinching the aorta and the pulmonal artery during one minute, while trpb was infused continuously in a cervical vein, a considerable passage of dye was observed in the CNS. This fact perhaps explains the discussed passage of dye in small animals (with or without injection of adrenaline). It may be that the injected quantity of dye-solution in connection with its injurious effect on the heart raises the venous pressure above a limit which is dangerous for some of the intracerebrospinal veins. Perhaps the passage of dye out into the CNS in bigger animals (rabbits), too, in experiments with insulin-, pentamethylene tetrazole- and electro-shock, found by BJERNER, BROMAN and SWENSSON (1944), is caused by a similar mechanism (the Valsalva effect).

V. Experiments with histamine.

It is a well-known fact that histamine has a remarkably injurious effect on the permeability of different vessels, resulting in the passage of plasma proteins. Therefore the negative results in our experiments on the cerebral vessels seem rather noteworthy. They obviously react differently to other vessels towards histamine.

VI. Experiments with allergic lesions.

Many attempts have been made by several investigators to produce allergic lesions of the CNS in experimental animals. Different methods have been used to indicate the lesion. In some cases only the symptoms of the animals have been registered — a very doubtful method. In other cases histological examinations were made and signs of hyperergic reactions looked for. Up to now no one but SKOOG has used the colour indicator method to demonstrate a disturbed permeability function of the cerebral vessels in the injured area.

The ways of causing the lesion have also differed. Symptoms of anaphylactic shock were earlier regarded as more or less due to a local allergic reaction in the brain. Later on their peripheral origin was established. Microscopic investigations (STIEF and TOKAY 1934, 1937) have proved the existence of minute bleedings and cellular injuries in the brain after anaphylactic shock. Our investigations (table 5 a) show that there is no disturbance of the cerebral vascular barrier-function during an anaphylactic shock. It therefore seems probable that the above-mentioned microscopic findings just as some of the symptoms are signs of secondary cerebral lesions caused by general circulatory disturbances and defective oxygen-saturation of the blood and thus do not indicate an allergic reaction localized to the CNS. That they cannot be the result of histamine release is proved above.

Later investigators have used special methods in order to localize the allergic reaction in the brain. Thus antigen (in sensitizing as well as in reacting doses) has been introduced intrathecally or directly into the brain substance by injection or implantation. In some of our experiments the antigen was also injected into a cerebral artery. Thus all kinds of mechanical measures which may be of localizing significance seem to have been tried. The results can be summarized as follows:

In experiments where the reacting dose was injected into the brain symptoms of cerebral lesion and microscopic changes which suggest a hyperergic reaction were found by DAVIDOFF, SEEGAL and SEEGAL (1932), KOPEROFF, DAVIDOFF and KOPEROFF (1936), ALEXANDER and CAMPBELL (1937). Controls with injection of an inactive substance elicited very weak symptoms. The above investigators concluded that a local allergic reaction had been caused in the brain. Our investigations (table 5 c) seem to confirm this conclusion, as in most cases an extensive zone of disturbed permeability was observed round the place of injection.

In experiments where the sensitizing dose of antigen was implanted in the brain in collodium-sacs or in pieces of agar and the reacting dose was administered intravenously DAVIDOFF and KOPELOFF (1931) and DAVIDOFF, KOPELOFF and KOPELOFF (1935) were able to demonstrate transient symptoms of disturbed cerebral function. After the primary operation similar symptoms (paresis) had been observed, caused by the mechanical lesion but they disappeared after a while. They concluded that the new wave of symptoms caused by the reacting dose was the result of a local allergic reaction in the brain. Unfortunately no microscopic examination was made. In our experiments with the usual antigens (horse-serum and ovalbumin) we found no disturbance of the barrier-function by injection of the reacting dose, unless the brain was mechanically injured at the same time (tables 5 a, b and c). We are, therefore, not able to confirm the above-mentioned opinion that a local allergic reaction can be produced in the brain in this way. The discrepancy may be due to the fact that we did not use their method of sensitizing the animals through implantation of the antigen into the brain. It is, however, notable that their animals showed at the same time symptoms of general anaphylaxis, and a possible latent lesion of the brain from the primary operation is very likely to become manifest in such a condition; the symptoms observed must not without further evidence be interpreted as due to a local allergic reaction.

If our conception is right, local allergic reactions cannot be produced in the brain by means of the ordinary laboratory antigens, unless there is at the same time a cerebral lesion, which offers a passage for substances from the blood into the injured part of the brain. Intracerebral injections of antigens thus open the way for antibodies from the blood which enter the brain substance. Except for the method of reverse allergy, this seems to be the only way of producing an antigen-antibody-reaction in the CNS. A similar mechanism may be involved in cases of infectious granulomas, only with the difference that the antigens are then produced locally. Provided that the allergic reaction increases the vascular permeability, a vicious circle is established and the process is limited only by the quantity and the diffusion of the antigens.

Experiments concerning reverse allergic reactions in the brain were first carried out by FORSSMAN (1922, 1926) and by FORSSMAN and SKOOG (1925). They used guinea-pigs and injected sheep-

hemolytic rabbit serum which contains antibodies against an antigen that is supposed to occur normally in the tissues of the guinea-pig. If injected intravenously this serum induces an anaphylactic shock in the animal. After injection into the carotid artery there will appear symptoms of cerebral lesion. INGVAR (1927) found by microscopic examination damage of nerve cells and small bleedings in the engaged part of the brain. SKOOG (1937, 1939) demonstrated by means of the colour indicator method that the lesion is combined with a pronounced disorder of the barrier-function.

The assumption that this lesion is of allergic origin has been specially emphasized by SKOOG. We also found this opinion reasonable, but we considered it advisable to make it a subject of closer analysis. The results of our experiments can be summarized as follows:

1) In no case direct application of rabbit serum on the pial vessels caused a disturbance of the barrier-function.

2) Intravascular administration of rabbit serum diluted in saline solution, guinea-pig's plasma or serum (complement present!) had also no injurious effect on the BBB, while in the presence of guinea-pig's blood corpuscles, rabbit serum caused a marked disturbance of the barrier-function.

3) This disturbance could be localized to any part of the brain according to the mode of injection.

From this we conclude that the cerebral lesion is not a direct manifestation of an antigen-antibody-reaction occurring in the nerve tissue or in the vascular wall, and it may be questioned if it is of allergic origin at all. Whatever kind of reaction it may be, the presence of guinea-pig's blood corpuscles seems to be a necessary condition for it to take place. That this reaction occurs only if the endothelial layer of the vessels is exposed to the agent, agrees with our conception that the endothelium constitutes the barrier-membrane. It seems probable that the microscopic alterations in the brain are secondary to the disorder of the barrier-function. The observation in point 3 indicates — contrary to SKOOG's assumption — that no part of the brain is more susceptible than others in this respect.

Our explanation clears up certain obscure points in earlier research-work. Thus it becomes understandable that precautions must be taken at the injection so that the rabbit serum mixes with the guinea-pig's blood before reaching the brain. For this purpose an

optimal rate of injection is necessary. When the injection was made in the cranial (distal) part of the ligated carotid artery, FORSSMAN and SKOOG obtained negative results, which were confirmed in our experiments with perfusion of the brain hemisphere in the absence of guinea-pig's blood corpuscles (table 4 c). The negative results by perfusion of anterior parts of guinea-pigs with Ringersolution shortly after death (BROMAN 1941) are explained in the same way.

Summary.

The permeability of the cerebrospinal vessels has a specific characteristic — the existence of a so-called blood-brain-barrier (BBB). Lesions of the vessels, causing a disorder of the barrier-function, can be demonstrated by a colour indicator method: Certain dyes, *e.g.* trypan blue, which cannot normally penetrate the vascular wall, are observed to pass, when the barrier-function is deranged.

The present investigation is designed to be a crude survey of the disorders of the barrier-function elicited by some chemical and physico-chemical agents. The experiments have been performed on guinea-pigs, rabbits and cats. After the different ways in which the substances have been made to act on the cerebral vessels the experiments have been arranged into three series:

- Series 1: Injection into the circulating blood in a cerebral artery; effective concentration unknown due to dilution; *post mortem* control of the passage of the dye.
- Series 2: Local application on the pial vessels; effective concentration known; control *in vivo* of the passage of the dye.
- Series 3: Perfusion during a short period of time (usually 1 min.) of the cerebral vessels with a test-solution; effective concentration known; control of the passage of the dye *in vivo* (inspection of the pial vessels) and *post mortem*.

Table 6.

Results (the injurious minimum concentrations in series 2 and 3).

Agents	Disorder of the barrier function		
	Series 1	Series 2	Series 3
Aqua dest.	—	— (10 minutes)	+ (2-3 minutes)
Hypertonic salt solution	+ (20%)	+ 8%	+ 5%
Acid solutions		- pH 1	+ pH 4

Agents	Disorder of the barrier function		
	Series 1	Series 2	Series 3
Alkaline solutions		+ pH 10—11	+ pH 10
Na-glycocholate	+ 10 %	+ 5 %	+ 0.5 %
Na-desoxycholate	+ 2 %	+ 2.5—5 %	+ 0.4 %
Ethyl alcohol	+ 70 %	+ 40—50 %	+ 15 %
Diphtheria toxin	—	—	
Tetanus toxin	—	—	
Staphylococcus toxin	—	—	
Cobra venom	+ 0.7 %	+ 10 %	+?
Bee venom	+ 1 %		
Adrenaline	—	—	
Acetylcholine	—	—	
Histamine	—	—	
Horse serum and ovalbumine in attempts to produce aller- gic reactions	—	—	
Sheep-hemolytic rabbit serum in guinea-pigs	+	—	
Witte peptone	—	—	+

(Substances which gave negative results in series 1 and 2 were not tested in series 3.)

For practical reasons the tested substances have been allowed to exert their influence only for a short period of time. This undoubtedly represents a source of error when estimating the injurious minimum concentrations.

The fact that the noxious substances disturb the barrier-function in significantly lower concentrations if administered on the intima-side of the vessels than on the adventitia-side agrees with the conception of the intima membrane as the site of the permeability — including the BBB-function.

It is obvious that certain substances have an injurious influence on the nerve cells in concentrations below those needed for disturbing the barrier-function. That applies to hypotonic salt solutions (aq. dest. in short-time experiments) and to ethyl alcohol. Other substances seem first to destroy the vascular membrane, thereby causing secondary lesion of the nervous tissue. That appears to apply to cobra venom and to sheep-hemolytic rabbit serum in experiments with guinea-pigs.

Changes of the osmotic pressure and of the hydrogen ion concentration cause a disorder of the barrier-function only if they are fairly great. From a methodological point of view this is of value in experiments with other substances.

The negative results in experiments with histamine as well as

the positive results in those with bile salts and ethyl alcohol may have a certain clinical interest.

It seems probable that proteins (toxins, venoms, antigens) cannot pass from the blood into the normal brain, if they do not exert a toxic influence on the vessels. A disturbed permeability may then render their passage possible (if they are still circulating in the blood), but is in itself reason enough for a secondary injury to the brain tissue.

A local allergic reaction in the brain with disturbance of the barrier-function can seemingly be produced by antigens such as horse serum only if they are injected intracerebrally, thus causing a mechanical injury, which allows a passage of antibodies from the blood into the brain. In the FORSSMAN-SKOOG-experiments a cerebral lesion of probably allergic origin (reverse allergy) is also elicited. Our experiments have shown, however, that this lesion is not caused by a simple reaction between the injected antibodies and the vascular endothelium, but that the blood corpuscles of the animal must contain a third factor necessary for the reaction.

Literature.

ALEXANDER, L., and A. C. P. CAMPBELL, Amer. J. Path. 1937. *13*. 229.
 BELLAVITIS, C., Riv. Path. nerv. 1929. *34*. 347.
 BJERNER, B., T. BROMAN and Å. SWENSSON, Acta psych. neurol. 1944. *19*. 431.
 BROMAN, T., Skand. Arch. Physiol. 1938. *80*. 59.
 —, Arch. Psychiatr. 1940. *112*. 290. 309.
 —, Acta path. microbiol. 1940. Suppl. 42.
 —, Acta psych. neurol. 1941. *16*. 1.
 —, Acta physiol. scand. 1941. *2*. 83.
 —, Acta med. scand. 1944. *118*. 79.
 DAVIDOFF, L. M., and N. KOPELOFF, Proc. Soc. exp. Biol., N.Y. 1931. *29*. 71.
 DAVIDOFF, L. M., B. C. SEEGAL, and D. SEEGAL, J. exp. Med. 1932. *55*. 163.
 DAVIDOFF, L. M., N. KOPELOFF, and L. M. KOPELOFF, J. Lab. clin. Med. 1935. *20*. 1238.
 FORSSMAN, J., Biochem. Z. 1922. *133*. 114.
 —, Acta path. microbiol. 1926. *3*. 749.
 FRIEDEMANN, U., and A. ELKELES, Klin. Wschr. 1932 II. 2026.
 —, Lancet 1934: I. 719. 775.
 GOLDMANN, E., Vitalfärbung am Zentralnervensystem, Berlin 1913.
 INGVAR, S., Acta path. microbiol. 1927. *4*. 349.
 KOPELOFF, N., L. M. DAVIDOFF and L. M. KOPELOFF, Proc. Soc. exp. Biol., N.Y. 1936. *34*. 155.

KROGH, A., personal communication (will be published 1945).

MACKLIN, C. C., and M. T. MACKLIN, Arch. Neurol. Psychiat. 1920. 3. 353.

MENDEL, W., Z. ges. Neurol. Psychiat. 1928. 117. 148.

RISER, Le Liquide Céphalo-Rachidien, Paris. 1929.

RUSSEL, D. S., Amer. J. Pathol. 1929. 5. 451.

SCHMID, H., Arch. Psychiatr. 1931. 95. 303.

SKOOG, T., Acta otolaryng. Stockh. 1937. 25. 365.

—, Ibidem 1939. Suppl. 32.

SPATZ, H., Allg. Z. Psychiatr. 1924. 80. 285.

—, Arch. Psychiatr. 1931. 101. 267.

STIEF, A., and L. TOKAY, Z. ges. Neurol. Psychiat. 1934. 150. 715.

—, Psychiatr. Neurol. Wschr. 1937. 39. 191.

WALLACE, G. B., and B. B. BRODIE, J. Pharmacol. 1937. 61. 397.

(Aus dem Pharmakologischen Institut der Universität Lund.)

Gefässerweiternde Stoffe des Harns und Renin.¹

Von

ERNST WOLLHEIM.

Eingegangen am 14. Juni 1945.

Im Harn des Menschen und der bisher untersuchten Tiere werden zwei gefässerweiternde, durch Cellophan nicht dialysable Stoffe in erheblichen Mengen ausgeschieden: das thermolabile Kallikrein, das bei Erhitzung auf 60 bis 70° seine Wirkung verliert (FREY, KRAUT u. Mitarb., 1926, 1928, 1930, 1934), und das thermostabile Depressan (WOLLHEIM, 1932, 1936, 1937). Diese beiden Substanzen passieren auf dem Wege von ihren Bildungsstätten durch die Blutbahn in den Harn die Nieren. Wenn derartige gefässerweiternde Stoffe normalerweise durch die Nieren ausgeschieden werden, liegt die Frage nahe, wie sie sich zu dem von TIGERSTEDT u. BERGMAN 1898 in der Niere gefundenen, eine Blutdrucksteigerung auslösenden Renin verhalten, dessen Bedeutung für die experimentelle Hypertonie und den Hochdruck des Menschen während der letzten Jahre in zahlreichen Arbeiten diskutiert wurde (vergl. u. a. PAGE, 1939, 1940, EULER, 1942). Die Versuche, über die hier berichtet werden soll, beschäftigen sich mit den Beziehungen zwischen den gefässerweiternden Stoffen des Harns und Renin.

Versuchsergebnisse.

Eine erste Orientierung über das Problem gibt folgender Versuch: Infundiert man einem Kaninchen in Urethan-Narkose (1.5 g pro kg intramusk.) langsam Depressan intravenös, so sinkt der

¹ Nach einem Vortrag in Fysiologföreningarna, Stockholm, am 6. Oktober 1944.

arterielle Blutdruck je nach der Infusionsgeschwindigkeit auf ein mehr oder weniger tiefes Niveau ab, auf dem er während der Infusionsdauer verharrt. Wählt man die Infusionsmengen nicht zu gross, so kann der Blutdruck nach Beendigung der Infusion allmählich im Laufe von etwa 1 Std. wieder auf das Ausgangsniveau ansteigen. Fig. 1 A zeigt einen derartigen Versuch, bei dem 0.6 mg eines Depressanpräparates pro Minute einem Kaninchen von 2.2 kg infundiert wurden. Vor Beginn der Infusion wurde die blutdrucksteigernde Wirkung der intravenösen Injektion von 5 γ Adrenalin geprüft. Diese Adrenalinwirkung ist während der Depressan-Infusion unverändert. Dagegen wirkt die intravenöse Injektion von 0.3 bis 0.5 ml Renin während der Depressan-Infusion kaum oder gar nicht blutdrucksteigernd. Die gleiche Erfahrung konnte auch an Katzen (in Pernocton-Narkose) gemacht werden. Dabei ist die Empfindlichkeit von Katzen gegen Depressan bei Anwendung gleicher Gewichtsmengen pro kg Versuchstier meist wesentlich schwächer, als die von Kaninchen, während die entsprechenden Renin-Mengen bei Katzen stärker blutdrucksteigernd wirken. Trotzdem wird bereits bei einer verhältnismässig geringen Blutdrucksenkung während der Depressan-Infusion der blutdrucksteigernde Effekt des injizierten Renins fast oder vollkommen aufgehoben.

Ebenso wie es EULER u. SJÖSTRAND (1943) bei Katzen beobachteten, konnte auch bei Kaninchen festgestellt werden, dass gelegentlich einzelne Versuchstiere schon bei der ersten Renin-Injektion, also ohne jeden weiteren Eingriff und vor der Möglichkeit einer sog. Renin-Tachyphylaxie, gegen Renin vollkommen refraktär waren, ohne dass ein Grund hierfür erkennbar wurde. Negative Befunde bezüglich der Renin-Wirkung sind daher nur beweisend, wenn sie an einer Serie von Tieren festgestellt werden. Das gleiche gilt auch für die weiter unten zu besprechenden Versuche, bei denen jede der Präparationen stets an drei verschiedenen Kaninchen auf ihre Blutdruckwirkung geprüft wurde.

Die für die Versuche dieser Arbeit angewendeten *Depressan*-Präparate (= »Vadil Ciba») hatten einen verschiedenen Reinheitsgrad. Alle Präparate waren aber wesentlich mehr von unwirksamen Begleitstoffen befreit, als die in meinen früheren Veröffentlichungen benutzten (1932, 1936 und 1937). Geprüft an der Blutdruckwirkung auf etwa 2 kg schwere Kaninchen in Urethan-Narkose hatten die alten Präparate (1936, 1937) bei intravenöser Injektion eine Schwellenwertswirkung mit 1 bis 5 mg pro kg

Versuchstier. Die jetzt zur Verfügung stehenden Präparate haben eine Schwellenwertsdosis von 0.01 bis 0.05 mg pro kg, zum Teil noch weniger. Eine mittlere und langanhaltende Blutdrucksenkung (30 bis 50 mm Hg für 30 bis 60 Minuten) wird durch 0.2 bis 1.0 mg pro kg bewirkt. Dieser Dosis efficax gegenüber ist die letale Dosis 30 bis 50 mg pro kg, die therapeutische Breite also

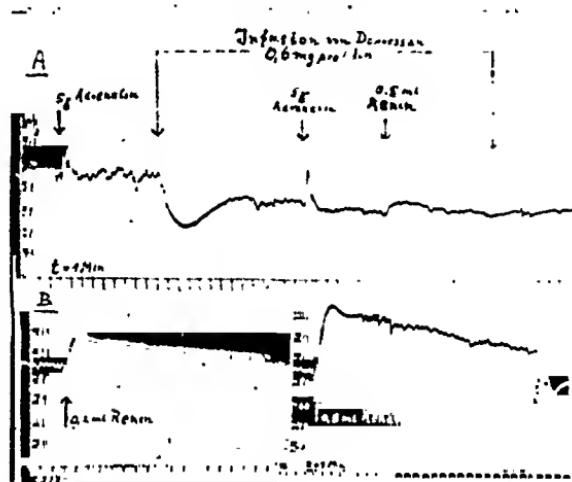


Fig. 1. Kaninchen, 2.2 kg, Urethan-Narkose, Blutdruck Art. Carotis.

- A (oben): Intravenöse Infusion eines Depressanpräparates, 0.6 mg pro Minute. Intravenöse Injektion von 5 γ Adrenalin vor Beginn und während der Depressaninfusion. 0.5 ml Renin intravenös während der Depressaninfusion.
- B (unten): Intravenöse Injektion von 0.2 und 0.5 ml Renin bei zwei anderen Kaninchen.

sehr gross. Fig. 2 zeigt die Wirkungen von 0.1 und 1.0 mg eines der Präparate.

Das in allen Versuchen verwendete *Renin*-Präparat wurde mir freundlicherweise von Professor ULF VON EULER zur Verfügung gestellt, dem ich auch an dieser Stelle nochmals herzlich danken möchte. Es war ein sehr gut haltbares Präparat aus Schweineniere, das seine Wirksamkeit während fast 2 Jahren kaum veränderte. Beim Kaninchen bewirken 0.2 bis 0.5 ml dieses Renins die bekannte langdauernde Blutdrucksteigerung um 30 bis 40 mm Hg (s. Fig. 1 B).

Man fragt sich, ob der geschilderte Befund, die Herabsetzung bzw. völlige Aufhebung der blutdrucksteigernden Wirkung von Renin durch die gleichzeitige Infusion von Depressan — im Gegensatz zu dem vollkommen unbeeinflussten Blutdruckeffekt von Adrenalin — durch einen Antagonismus am Erfolgsorgan hervorgerufen wird, oder wie das Phänomen sonst zu Stande kommt.

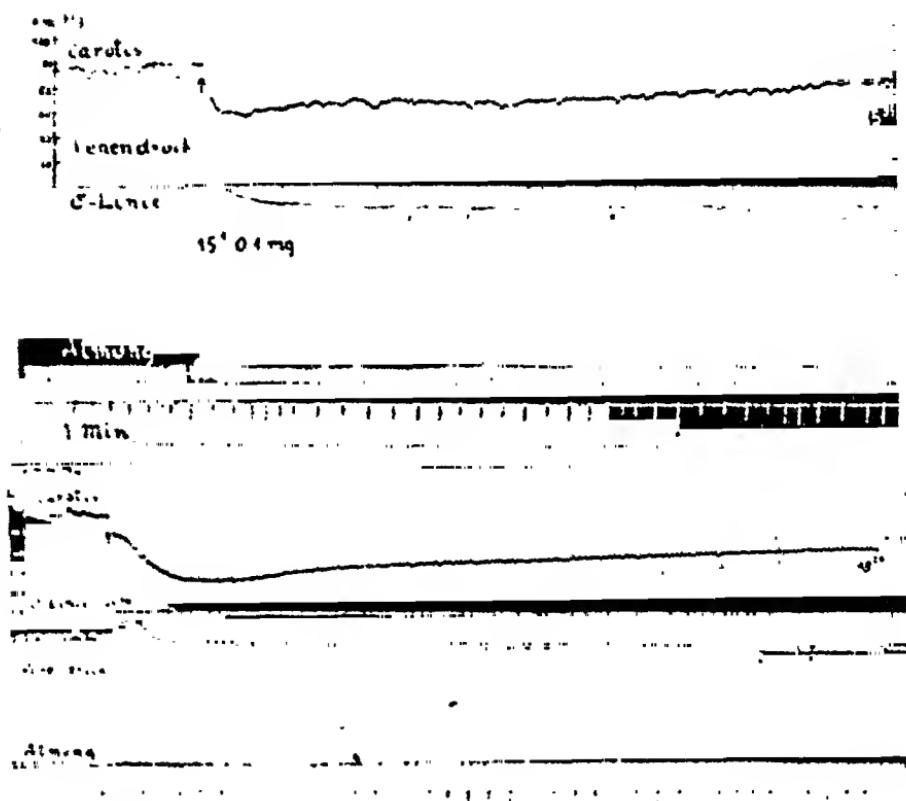


Fig. 2. Kaninchen, 2.1 kg, Urethan-Narkose, Blutdruck Art. Carotis, Venendruck und Atmung.

Oben: Intravenöse Injektion von 0.1 mg Depressan (Vadil + Ciba).
Dauer der Blutdrucksenkung 34 Minuten.

Unten: Intravenöse Injektion von 1 mg des gleichen Präparates.
Dauer der Wirkung mehr als 50 Minuten.

Renin wirkt bekanntlich selbst nicht direkt vasokonstriktorisch. Wie fast gleichzeitig und unabhängig von einander MUÑOZ, BRAUN-MENENDEZ, FASCIOLI u. LELOIR (1939, 1940) sowie KOHLSTAEDT, HELMER u. PAGE (1938) zeigten, muss Renin auf einen Stoff im Bluts serum einwirken, als Hypertensinogen oder Renin-Aktivator bezeichnet, um einen vasokonstriktorischen Effekt auszulösen. Diese enzymartige Reaktion, in der wahrscheinlich das thermolabile Renin das Enzym, das Hypertensinogen resp. der Renin-Aktivator das Substrat ist, führt zu der Bildung der eigentlichen blutdrucksteigernden thermostabilen Substanz, von BRAUN-MENENDEZ u. Mitarb. (1939, 1940) Hypertensin, von PAGE u. HELMER (1940) Angiotonin genannt.¹

¹ Hypertensin und Angiotonin sind zweifellos identisch, ebenso Hypertensinogen und der Renin-Aktivator. Im folgenden werden nur die Bezeichnungen »Hypertensin« und »Hypertensinogen« benutzt.

Das *Hypertensinogen* ist in der Pseudoglobulin-Fraktion des Serums enthalten, die für die eigenen Versuche nach der Vorschrift von EDMAN, EULER, JORPES u. SJÖSTRAND (1942) durch Halbsättigung mit Ammoniumsulfat und anschliessende 48- bis 72-stündige Dialyse des Niederschlages in Cellophan gegen fliesendes Wasser aus Pferdeserum gewonnen wurde. Die Sera kamen unmittelbar nach der Entnahme in den Eisschrank und wurden nach spätestens 24 Std. weiter verarbeitet.

Der blutdrucksteigernde Stoff *Hypertensin* entsteht durch Inkubation von Renin mit dieser Pseudoglobulin-Fraktion des Serums bei 40° während 10 Minuten. Als zweckmässigstes Mengenverhältnis erwiesen sich 1 bis 2 Teile Renin auf 100 Teile Serum-Globulin. Eine Verdoppelung der Renin-Menge steigerte die Ausbeute an blutdrücksteigerndem Stoff nur wenig.

Die für die Reihenversuche dieser Arbeit angewandten Mengen waren im allgemeinen 0.2 bis 0.4 ml Renin zu 20 oder 40 ml Serum-Globulin. Die Inkubierung erfolgte bei pH = 7.2 bis 7.45 im Wasserbad (40°) in Zentrifugengläsern aus Jenaer Glas. Nach 10 Min. wurden die Gläser in ein kochendes Wasserbad überführt, in dem sie weitere 10 Min. verblieben. Die Temperatur der Lösung ist nach 3 Min. über 80° und steigt dann auf etwa 90°. Hierdurch wird etwa noch vorhandenes freies Renin zerstört, während das gebildete Hypertensin kochbeständig ist. Nach dem Erhitzen, bei dem ein weisser Niederschlag ausfällt, wird 1/2 bis 1 Std. bei 3,000 Touren zentrifugiert. Um Hypertensin in Trockensubstanz zu erhalten, kann nach der von v. EULER u. Mitarb. gegebenen Vorschrift weiter verfahren werden (Alkoholextraktion, Aetherfällung), was aber grössere Mengen an Ausgangsmaterial voraussetzt, als 20 ml. Für die Fragestellung dieser Versuche erwies es sich als ausreichend, die nach dem Zentrifugieren überstehende klare Flüssigkeit direkt in Mengen von 1 bis 2 ml auf ihre Blutdruckwirkung am Kaninchen bei intravenöser Injektion zu prüfen. Aus 20.2 ml des ursprünglichen Ansatzes wurden so 8 bis 10 ml Hypertensin-Lösung gewonnen, die ohne Wirkungsverlust bis zu 1 Woche im Eisschrank haltbar ist. 1 ml dieser Lösung entsprach der Wirkung von 0.3 bis 0.5 mg Hypertensin, wie es nach dem Verfahren von U. v. EULER u. Mitarb. erhalten wurde. Eine Verdoppelung der Dosis bewirkt eine stärkere, aber nicht doppelt so starke Blutdrucksteigerung (vergl. Fig. 3). Eine weitere Steigerung der Dosis erhöht den Blutdruckeffekt nur unwesentlich, was sich mit den Erfahrungen anderer Autoren deckt.

Es war zu prüfen, ob die gefässerweiternden Stoffe des Harnes die Bildung von Hypertensin bei der Inkubation von Renin mit Serum-Globulin beeinflussen resp. verhindern.

Für diese Versuche wurden zunächst drei Präparate des thermostabilen Stoffes Depressan von verschiedenem Reinheitsgrad

verwendet: die blutdrucksenkende Wirkung von 2 mg des Präparates I entsprach der von 0.5 mg des Präparates II und von 1 mg des Präparates III (s. Fig. 4 A). Die für einen gleich starken Blutdruckeffekt notwendigen, blutdruckaequivalenten Gewichtsmengen verhielten sich also wie 1 : 1/4 : 1/2.

Setzt man dem Ansatz von 0.2 oder 0.4 ml Renin und 20 ml Serum-Globulin 20 mg des Präparates I zu, inkubiert in der oben beschriebenen Weise 10 Min. bei 40°, erhitzt danach während 10 Min. auf etwa 90° und zentrifugiert, so erweist sich das resul-

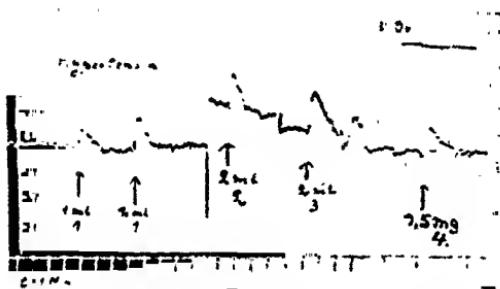


Fig. 3. Kaninchen, ca. 2 kg, Urethan-Narkose, Blutdruck Art. Carotis, bei der letzten Injektion auch Registrierung des Venendruckes. Intravenöse Injektion von 1 bzw. 2 ml vier verschiedener Hypertensin-Präparate.

- 1: Hypertensin aus 40 ml Pferdeserum + 0.4 ml Renin.
- 2: Hypertensin aus 40 ml Serum-Globulin + 0.4 ml Renin.
- 3: * * * * * + 0.8 ml *
- 4: Hypertensin aus 100 ml Serum-Globulin + 2 ml Renin, 0.5 mg in 2 ml. Aq. dest.

tierende Reaktionsprodukt in Mengen von 1 bis 2 ml am Kaninchenblutdruck als unwirksam (s. Fig. 4 B, Injektion Nr. 1). Nimmt man erheblich kleinere Mengen des Depressan-Präparates, so erhält man eine Lösung, die den gewöhnlichen blutdrucksteigernden Effekt des Hypertensins zeigt. Setzt man dem Ansatz grössere Mengen des Präparates I zu, 30 oder 40 mg, so resultiert eine Lösung mit blutdrucksenkender Wirkung. Die kleinsten Mengen der beiden anderen Depressan-Präparate, die eine Aufhebung des blutdrucksteigernden Hypertensin-Effektes bewirken, sind 5 mg Präparat II und 10 mg Präparat III (s. Fig. 4 B, Injektionen Nr. 3 und 5). Diese absoluten Mengen der gefässerweiternde Stoffe wurden an allen geprüften Pferde-Seren, bzw. Serum-Globulinen mit Ausnahme eines gefunden. Bei letzterem waren von allen drei Depressan-Präparaten etwas grössere Mengen erforderlich, um die blutdrucksteigernde Hypertensin-Wirkung aufzuheben. Aber stets entsprachen die von den drei Präparaten

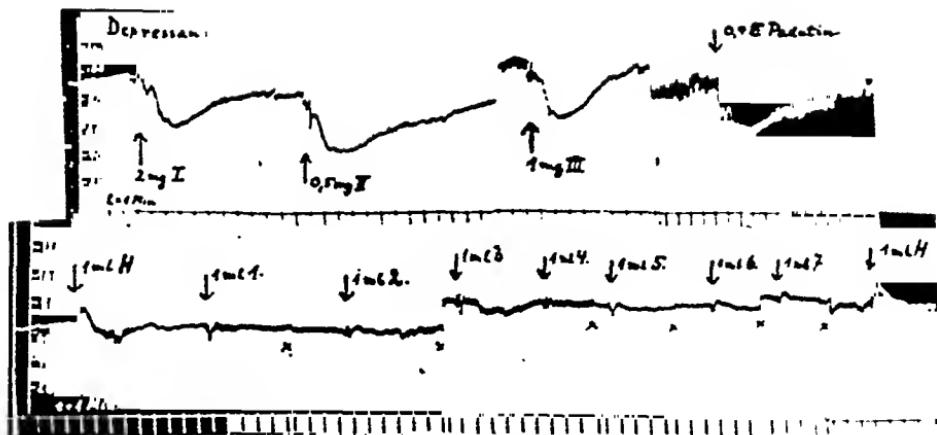


Fig. 4. Kaninchen, ca. 2 kg, Urethan-Narkose, Blutdruck Art. Carotis.

A (oben): Intravenöse Injektion von Depressan: 2 mg Präparat I, 0.5 mg Präparat II, 1 mg Präparat III; sowie 0.4 E Kallikrein (= "Padutin").

B (unten): Intravenöse Injektionen von je 1 ml:

H: Hypertensin aus 20 ml Serum-Globulin + 0.4 ml Renin.

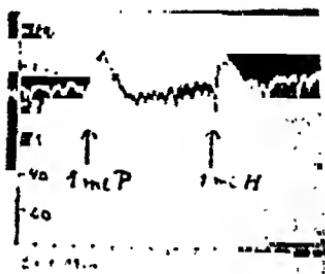
- 1: 20 ml Serum-Globulin + 0.4 ml Renin + 20 mg Depressan I, 10 Min. bei 40° inkubiert, auf 90° erhitzt, zentrifugiert.
- 2: 20 ml Serum-Globulin + 20 mg Depressan I, 10 Min. bei 40° inkubiert, dann Zusatz von 0.4 ml Renin, weitere 10 Min. bei 40° inkubiert, auf 90° erhitzt, zentrifugiert.
- 3: 20 ml Serum-Globulin + 0.4 ml Renin + 5 mg Depressan II, 10 Min. bei 40° inkubiert, auf 90° erhitzt, zentrifugiert.
- 4: 20 ml Serum-Globulin + 5 mg Depressan II, 10 Min. bei 40° inkubiert, dann Zusatz von 0.4 ml Renin, weitere 10 Min. bei 40° inkubiert, auf 90° erhitzt, zentrifugiert.
- 5: 20 ml Serum-Globulin + 0.4 ml Renin + 10 mg Depressan III, 10 Min. bei 40° inkubiert, auf 90° erhitzt, zentrifugiert.
- 6: 20 ml Serum-Globulin + 10 mg Depressan III, 10 Min. bei 40° inkubiert, dann Zusatz von 0.4 ml Renin, weitere 10 Min. bei 40° inkubiert, auf 90° erhitzt, zentrifugiert.
- 7: 20 ml Serum-Globulin + 0.4 ml Renin + 5 mg Depressan III, 10 Min. bei 40° inkubiert, auf 90° erhitzt, zentrifugiert.

notwendigen Mengen dem Verhältnis ihrer blutdrucksenkenden Wirkung, ihren Blutdruckaequivalenten (s. Fig. 4 A und 4 B).

Als thermolabiler gefässerweiternder Stoff (Kallikrein) wurde in gleicher Weise ein Handelspräparat »Padutin Bayer» geprüft. 0.1 ml = 0.4 E¹ dieses Präparates entsprachen in ihrer blutdrucksenkenden Wirkung am Kaninchen ungefähr dem Effekt von 2 mg des Depressan-Präparates I, von 0.5 mg des Präparates II und 1 mg des Präparates III (s. Fig. 4 A). Das Padutin wurde vorher 48 St. in Cellophan gegen fliessendes Wasser dialy-

¹ Als 1 E Kallikrein wurde von KRAUT, FREY u. BAUER (1928) die Menge definiert, die bei intravenöser Injektion am Hund die gleiche maximale Steigerung der Pulsumplitude bewirkt, wie 0.5 mg eines Standardpräparates. Das verwendete Handelspräparat Padutin enthält 4 E in 1 ml.

siert, und hierdurch von seinem Trikresolzusatz befreit. Während von den drei Depressan-Präparaten jeweils etwa das 10-fache der in ihrer Blutdruckwirkung in Fig. 4 A dargestellten Mengen notwendig war, um bei Zusatz zu einem Ansatz von 20 ml Serum-Globulin und 0.2 oder 0.4 ml Renin den blutdrucksteigernden Effekt des Reaktionsproduktes vollkommen zu verhindern (s. Fig. 4 B), erwies sich die 10-fache, ja sogar die 40-fache Menge Padutin in dieser Beziehung als unwirksam: Bei Zusatz von 16 E Padutin zu dem gleichen Ansatz von Serum-Globulin und Renin, Inkubierung während 10 Min. bei 40° und anschliessendem Erhitzen



Erhitzen 0.1 ml = 0.4 E (s. Fig. 6). Diese Prüfung setzt die oben erwähnte Dialyse des Präparates voraus, da Trikresol, wie auch einige andere Stoffe, die Zerstörung des Kallikreins durch Erhitzen hemmt (KRAUT, FREY u. WERLE, 1930, 1934). Geht man von dem kochbeständigen Rest der blutdrucksenkenden Wirkung des benutzten Padutin-Präparates aus, so war es wieder etwa die 10-fache Menge des Blutdruckaequivalentes aus Fig. 4 A und 6, die notwendig ist, um bei Zusatz zu dem Ansatz von 20 ml Serum-Globulin und 0.2 bzw. 0.4 ml Renin den blutdrucksteigernden Hypertensin-Effekt zu verhindern. Aus diesen Mengenverhältnis-

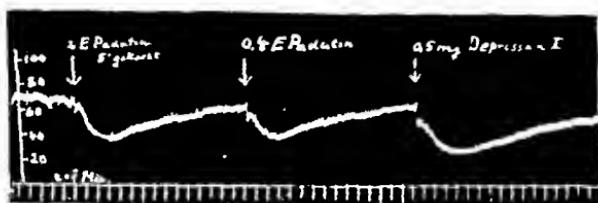


Fig. 6. Kaninchen, 1.9 kg, Urethan-Narkose, Blutdruck Art. Carotis. Intravenöse Injektionen von 0.5 ml = 2 E Padutin (48 Std. dialysiert), 5 Min. gekocht; 0.1 ml = 0.4 E Padutin (48 Std. dialysiert), ungekocht; 0.5 mg Depressan II.

sen geht hervor, dass das thermolabile Kallikrein selbst in dieser Beziehung unwirksam ist, die Wirkung der grossen Mengen von Padutin vielmehr nur der Beimengung von koktostabilem gefässerweiterndem Stoff entspricht, und demnach durch letzteren bedingt sein dürfte.

Als Kontrollversuch zu der spezifischen Einwirkung des Depressans auf den Inkubationsansatz von Renin und der Pseudoglobulin-Fraktion des Serums wurden noch andere gefässerweiternde Stoffe in der gleichen Versuchsanordnung untersucht. Fig. 7 zeigt einen derartigen Versuch mit dem esterasestabilen Carbaminoylcholinchlorid (»Doryl Merck«). Bei Zusatz von 40 μ Doryl zu 20 ml Serum-Globulin, Inkubierung bei 40° während 10 Min. und anschliessender Erhitzung auf etwa 90°, bleibt der blutdrucksenkende Effekt des Doryls annähernd unverändert. Wird die gleiche Dorylmenge einem Ansatz von 20 ml Serum-Globulin und 0.2 ml Renin zugesetzt und, wie oben beschrieben, weiter behandelt, so resultiert ein Produkt, von dem 1 und 2 ml am Kaninchen eine kurzfristige Blutdrucksenkung und Blutdrucksteigerung, aber keinen typischen blutdrucksteigernden Hypertensin-Effekt hervorrufen. Injiziert man aber 2 ml dieses Reaktionsproduktes nach vorheriger Atropinierung der Ver-

suchstiere (5 mg Atropin, sulf. intravenös), so findet man allein den charakteristischen Blutdruckanstieg, wie er durch Hypertensin ausgelöst wird. In Ansätzen mit Depressan oder Kallikrein verändert eine Atropinisierung die vorher beobachteten Effekte nicht. Im Inkubierungsansatz mit Doryl dagegen tritt der vorher durch die Summation einer blutdrucksenkenden und einer blutdrucksteigernden Wirkung verdeckte Effekt nach der Atropinisierung hervor, da durch diese die blutdrucksenkende Wirkung des Doryls aufgehoben wird. Doryl verhindert im Gegensatz zu

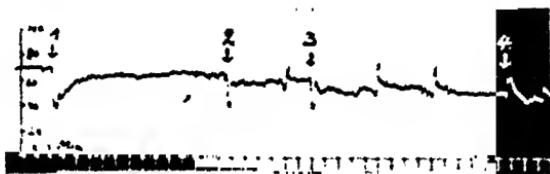


Fig. 7. Kaninchen, 2 kg, Urethan-Narkose, Blutdruck Art. Carotis. Intravenöse Injektionen.

1: 20 ml Serum-Globulin + 40 γ Doryl (Merck), 10 Min. bei 40° inkubiert, auf 90° erhitzt, zentrifugiert. 1 ml des Reaktionsproduktes injiziert.
 2—4: 20 ml Serum-Globulin + 0.4 ml Renin + 40 γ Doryl, 10 Min. bei 40° inkubiert, auf 90° erhitzt, zentrifugiert.
 2: 1 ml des Reaktionsproduktes injiziert.
 3: 2 * * *
 4: 2 * * * , nach 5 mg Atropin sulf. intravenös.

Depressan die blutdrucksteigernde Wirkung des Hypertensins nicht.

Die Frage liegt nahe, ob nicht Depressan bei seinem Zusatz zu den Inkubierungsansätzen den blutdrucksteigernden Effekt des Reaktionsproduktes auch nur deshalb aufhebt, weil sich in letzterem enthaltene pressorische und depressorische Stoffe in ihrer Wirkung summieren. Man könnte hieran vielleicht um so eher denken, als die Ansätze nach der Inkubierung bei 40° während 10 Min. auf 90° erhitzt wurden, und sich, wie oben gezeigt, nur das thermostabile Depressan, nicht aber das thermolabile Kallikrein als geeignet erwies, den blutdrucksteigernden Hypertensin-Effekt zu verhindern. Es zeigte sich aber, dass die für die Inkubierungsansätze benutzten Mengen der Pseudoglobulin-Fraktion des Serums ausreichen, um die blutdrucksenkende Wirkung der angewendeten Depressanmengen aufzuheben. Wie bekannt (KRAUT, FREY u. BAUER, 1928), wird Kallikrein in bestimmten Mengenverhältnissen durch Serum inaktiviert. Auch die blutdrucksenkende Wirkung von Depressan wird unter der Einwirkung von

Serum bei 40° mehr oder weniger aufgehoben. Vergleicht man die für die Inaktivierung blutdruckaequivalenter Mengen des thermolabilen und des thermostabilen Stoffes nötigen Mengen Serum, bei gleicher Temperatur und Einwirkungszeit, so zeigt sich, dass das Inaktivierungsvermögen des Serums gegenüber Kallikrein grösser ist, als gegenüber Depressan. Es ist hier nicht näher auf das Problem der Inaktivierung der beiden Stoffe einzugehen. Nur soviel ist zu konstatieren: die für die Inkubationsansätze mit Renin verwendeten Mengen Depressan (20 mg Präparat I, 5 mg

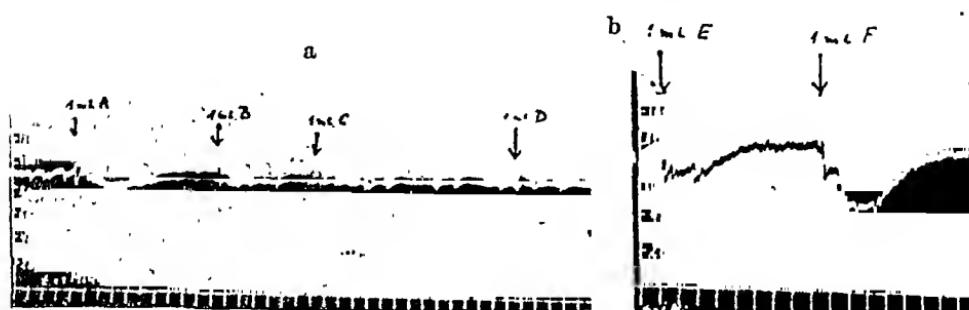


Fig. 8 a und b. Kaninchen, 1.9 resp. 2.0 kg, Urethan-Narkose. Blutdruck Art. Carotis. Intravenöse Injektionen von je 1 ml folgender Reaktionsprodukte, sämtlich 10 Min. bei 40° inkubiert, auf 90° erhitzt, zentrifugiert:

A: 20 ml Serum-Globulin + 8 mg Depressan II.
B: » » » + 20 » » I.
C: » » » + 10 » » III.
D: » » » + 5 » » II.
E: » » » + 10 » » II.
F: » » » + 20 » » II.

Präparat II, 10 mg Präparat III) verlieren unter den gleichen Bedingungen (10 Min. bei 40° und pH 7.2 bis 7.45) durch 20 ml der Pseudoglobulin-Fraktion des Pferde-Serums ihre blutdrucksenkende Wirkung vollkommen (s. Fig. 8 a). Setzt man der gleichen Menge Serum-Globulin grössere Mengen Depressan zu, z. B. 8 mg, 10 mg oder 20 mg Präparat II oder entsprechende Mengen der beiden anderen Präparate, so bleibt, je nach der Depressanmenge, ein blutdrucksenkender Effekt übrig (s. Fig. 8 b), wie es auch nach Zusatz dieser grösseren Depressanmengen zu den Inkubationsansätzen mit Renin beobachtet wurde.

Aus diesem Verhalten des thermostabilen Stoffes geht hervor: 1) Der sog. »Serum-Inaktivator« des Depressans befindet sich in der gleichen Pseudoglobulin-Fraktion des Pferdeserums, wie das Hypertensinogen. 2) Das Fehlen der blutdrucksteigernden Wirkung der Reaktionsprodukte aus Renin und Serum-Globulin bei Zu-

satz von Depressan beruht nicht auf der Summation einer in ihnen enthaltenen pressorischen und depressorischen Komponente (wie es in den Versuchen mit Doryl gefunden wurde); da vielmehr gleichzeitig auch der blutdrucksenkende Effekt des Depressans verschwindet, ist hier tatsächlich jede blutdrucksteigernde Wirkung aufgehoben.

Dass es sich dabei um eine Hemmung resp. Verhinderung der Bildung von Hypertensin aus Renin und Hypertensinogen unter

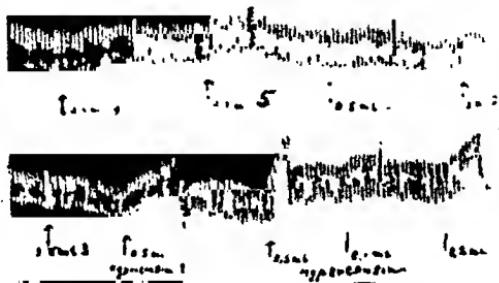


Fig. 9. Kaninchen-Dünndarm in Tyrode-Lösung, durchperlt mit $O_2 + 6\% CO_2$, Bad 50 ml. Zusatz von 0.5 ml der gleichen Reaktionsprodukte, die in Fig. 4 B auf ihre Blutdruckwirkung geprüft wurden.

1:	20 ml Serum-Globulin	+	0.4 ml Renin	+	20 mg Depressan I.		
3:	+	+	+	+	+	5 " "	II.
5:	+	+	+	+	+	10 " "	III.
7:	+	+	+	+	+	5 " "	III.
H:	+	+	+	+	+	+	III.

Sämtlich 10 Min. bei 40° inkubiert, auf 90° erhitzt, zentrifugiert.

6: 20 mg Serum-Globulin + 10 mg Depressan III, 10 Min. bei 40° inkubiert, dann Zusatz von 0.4 ml Renin, weitere 10 Min. bei 40° inkubiert, auf 90° erhitzt, zentrifugiert.
Rechts unten: 0.5 ml, 0.1 ml und 0.2 ml Hypertensin.

der Einwirkung des Depressans handelt, konnte durch eine weitere Versuchsreihe belegt werden. Am überlebenden Kaninchendarm (in 50 ml Tyrode-Lösung, durchperlt mit einem Gasgemisch von O_2 und 6 % CO_2) ruft Hypertensin in Mengen von 0.2 bis 0.5 ml eine Kontraktion hervor (s. Fig. 9), wie auch von PAGE (1940) gezeigt wurde. Die auf den Blutdruck unwirksamen Reaktionsprodukte, die mit Zusatz von Depressan inkubiert wurden (20 mg Präparat I, 10 mg Präparat III und 5 mg Präparat II zu einem Ansatz von 0.4 ml Renin und 20 ml Serum-Globulin), haben am Darm keine kontrahierende Wirkung (s. Fig. 9). Der Parallelismus der Wirkungen auf Blutdruck und Darm ist sehr weitgehend: Setzt man den Reaktions-Ansätzen etwas geringere Mengen Depressan zu, als zur völligen Aufhebung beider Wirkungen notwendig sind

(s. oben), z. B. 5 mg Präparat III, so entspricht einer geringen Blutdrucksteigerung eine schwache Kontraktion des Darms (s. Injektion Nr. 7 in Fig. 4 B und 9). Von den zu prüfenden Reaktionsgemischen wurden im allgemeinen 0.5 ml dem Darmbad zugesetzt, da Hypertensin in dieser Menge stark kontrahierend wirkt. Aber auch 1 ml der Präparationen ist unwirksam, wenn der Blutdruckeffekt aufgehoben ist (s. Fig. 10). Diese Versuchsreihe ist also ein weiterer Beweis dafür, dass der Zusatz bestimmter Mengen Depressan zu den Inkubierungsansätzen von Renin und Serum-Globulin die Hypertensin-Bildung verhindert.

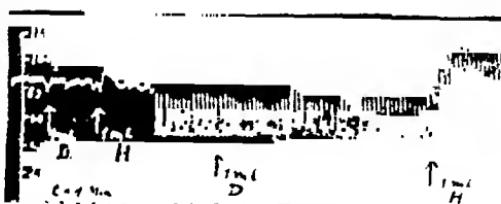


Fig. 10. Links: Kaninchen, 1.9 kg, Urethan-Narkose, Blutdruck Art. Carotis. Rechts: Kaninchendarm in Tyrodelösung (50 ml). Intravenöse Injektionen resp. Zusatz zum Darmbad je 1 ml.

D: 20 ml Serum-Globulin + 0.2 ml Renin + 5 mg Depressan II.

H: Hypertensin aus 20 ml Serum-Globulin + 0.2 ml Renin.

Beide Ansätze 10 Min. bei 40° inkubiert, auf 90° erhitzt, zentrifugiert.

Der gleiche Effekt ergab sich auch dann, wenn zunächst nur 20 ml Serum-Globulin mit den selben Mengen Depressan (20 mg Präparat I, 5 mg Präparat II oder 10 mg Präparat III) während 10 Min. bei 40° inkubiert und erst danach 0.4 ml Renin zugesetzt wurden. Die Ansätze wurden darauf weitere 10 Min. bei 40° inkubiert, auf etwa 90° erhitzt und zentrifugiert. Die so gebildeten Reaktionsprodukte, von denen 1 bis 2 ml auf ihre Blutdruckwirkung, 0.5 ml auf ihre Darmwirkung untersucht wurden, verändern den arteriellen Blutdruck der Kaninchen nicht (s. Fig. 4 B, Injektionen Nr. 2, 4 und 6) und sind am Kaninchendarm unwirksam (s. Fig. 9, Zusatz Nr. 6). Die Bildung von Hypertensin wird demnach durch den unmittelbar vorhergehenden Zusatz von Depressan ebenso verhindert, wie bei gleichzeitigem Zusatz von Depressan zu den Inkubierungsansätzen von Renin und Serum-Globulin.

Diskussion der Versuchsergebnisse.

Wie die geschilderten Versuche zeigen, verhindert der Zusatz des thermostabilen gefässerweiternden Stoffes aus Harn, des De-

pressans, zu den Inkubierungsansätzen von Renin und dem in der Pseudoglobulin-Fraktion des Serums enthaltenen Hypertensinogen die sonst unter gleichen Bedingungen erfolgende Bildung einer blutdrucksteigernden und darmkontrahierenden Substanz, des Hypertensins. Dass es sich bei diesem Effekt nicht nur um eine Summation der Wirkungen etwa im Reaktionsprodukt enthaltener blutdrucksteigernder und -senkender Stoffe handelt, geht daraus hervor, dass 1) die Reaktionsprodukte auch am Kaninchendarin unwirksam sind, der von Hypertensin erregt wird, während Depressan in den angewendeten Mengen keinerlei Wirkungen auf ihn ausübt, und dass 2) die blutdrucksenkende Fähigkeit der angewandten Depressanmengen durch die gleiche Pseudoglobulin-Fraktion des Serums unter den selben Bedingungen (Mengenverhältnisse, Einwirkungszeit, Temperatur und pH) aufgehoben wird.

Nach diesen Beobachtungen fragt man sich: Wie stört der Zusatz des Depressans die enzymartige Reaktion, die sonst zu der Bildung eines blutdrucksteigernden und darmkontrahierenden Stoffes aus der Einwirkung von Renin auf Serum-Globulin führt? Haben die beiden im Inkubierungsansatz möglichen Reaktionen — die Hypertensin-Bildung und die Depressan-Inaktivierung — engere Beziehungen zueinander, als nur die Anwesenheit des Hypertensinogens und des »Depressan-Inaktivators» in der gleichen Serum-Globulin-Fraktion?

Zu diesen Fragen ist festzustellen: In vivo, im Blutdruckversuch am Kaninchen verändert sich die blutdrucksenkende Wirksamkeit von Depressan nicht nach einer oder mehreren vorhergehenden Renin-Injektionen, auch wenn diese eine Renin-Tachyphylaxie hervorgerufen haben. Wie PAGE (1940) zeigte, haben solche Versuchstiere kein Hypertensinogen mehr in der Blutbahn. Da die Depressan-Wirkung unter diesen Umständen nicht verstärkt ist, spricht nichts dafür, dass durch den Verbrauch des Hypertensinogens die inaktivierende Fähigkeit des Serums gegenüber Depressan verändert wäre. In vitro, im Inkubierungsansatz mit Serum-Globulin, wird, wie gezeigt, durch gleichzeitig zugesetztes Renin die Inaktivierung des Depressans nicht verhindert, ja nicht einmal quantitativ verändert. Gleiche Mengen Depressan werden mit wie ohne Zusatz von Renin unter identischen Bedingungen (Zeit, Temperatur, pH) von gleichen Mengen Serum-Globulin inaktiviert. Daher kann nicht angenommen werden, dass das Hypertensinogen und der Inaktivator des Depressans das gleiche Substrat sind, auf

das etwa zwei verschiedene Enzyme einwirken, und bei einer gegebenen Menge des Substrates an ihm konkurrieren. Vielmehr müssen Depressan und Renin mit zwei verschiedenen Stoffen im Serum-Globulin reagieren, wobei Depressan die Reaktion zwischen Renin und Hypertensinogen verhindern; Renin aber die Inaktivierung des Depressans nicht hemmen kann.

Weiterhin zeigte sich, dass Depressan in den gleichen Mengenverhältnissen die Hypertensin-Bildung nicht nur bei gleichzeitigen Zusatz zu den Inkubationsansätzen von Renin und Serum-Globulin verhindert, sondern auch dann, wenn zunächst Depressan allein 10 Min. mit Serum-Globulin inkubiert, und erst danach Renin zugesetzt wurde, zu einer Zeit, in der das Depressan in Bezug auf seine Blutdruckwirkung bereits inaktiviert war. Man fragt sich daher, ob es überhaupt das Depressan selbst ist, das die Hypertensinbildung hemmt bzw. verhindert, oder nur ein Teil seines grossen Moleküls, oder etwa ein Begleitstoff, der mit der Blutdruckwirkung nichts zu tun hat.

Hierzu ist zu konstatieren: Bei gleichzeitig wie bei nacheinander erfolgendem Zusatz von Depressan und Renin sind stets die gleichen Mengen der drei untersuchten Depressan-Präparate von verschiedenem Reinheitsgrad notwendig, um eine Hypertensinbildung zu verhindern. Diese Mengen Depressan entsprechen bei ganz verschiedenen Gewichtsmengen der drei Präparate genau ihren Blutdruckäquivalenten (1 : 1/4 : 1/2). Dabei sind die zur Erreichung des Effektes nötigen Mindestmengen Depressan stets diejenigen, die maximal unter gleichen Bedingungen von den angewandten Mengen Serum-Globulin total inaktiviert werden können. Eine den benutzten Depressanmengen blutdruckäquivalente Menge des thermolabilen Stoffes aus Harn, des Kallikreins, verhindert die Hypertensinbildung nicht. Ein solcher Effekt trat dagegen auf, wenn die zugesetzten Mengen Padutin so gross waren, dass der Gehalt dieses Präparates an thermostabilem Stoff wiederum den wirksamen Depressanmengen blutdruckäquivalent war. Es kann daher nur angenommen werden, dass das Depressan bei gleichzeitigem wie bei vorhergehendem Zusatz zu den Inkubationsansätzen dem Hypertensinogen die Fähigkeit nimmt, unter der Einwirkung von Renin das blutdrucksteigernde Hypertensin zu bilden. Wie diese Einwirkung geschieht, kann noch nicht gesagt werden.

Für die vielfach diskutierte physiologische und pathologische Bedeutung des Renins dürften diese Befunde einige Kon-

sequenzen haben. Hier sei nur kurz auf folgendes hingewiesen:

1) In der normalen Niere befinden sich stets erhebliche Mengen von Depressan auf ihrem Ausscheidungsweg in den Harn. Hier sind also alle Vorbedingungen dafür gegeben, dass etwa in die Blutbahn abgegebenes Renin nicht zu der Bildung des blutdrucksteigernden Hypertensins führen kann.

2) Bei Patienten mit essentieller Hypertonie ist die Ausscheidung des thermostabilen gefässerweiternden Stoffes stark vermindert oder vollkommen aufgehoben (WOLLHEIM u. LANGE, 1932, ELLIOT u. NUZUM, 1934, WOLLHEIM, 1936, 1937). Bei diesen Kranken ist daher damit zu rechnen, dass auch in den Nieren weniger oder gar kein Depressan vorhanden ist. Das hier in die Blutbahn abgegebene Renin könnte also zu der Bildung von Hypertensin führen. Wenn auch aus verschiedenen Gründen Hypertensin nicht die alleinige chemische Ursache des erhöhten Blutdruckes sein kann, so könnte es doch einen zusätzlichen blutdrucksteigernden Faktor darstellen, eventuell für die Fixierung des hohen Blutdruckes bei länger bestehenden essentiellen Hypertonien und für die sich sekundär entwickelnden Nierenfunktionsstörungen solcher Patienten von Bedeutung sein.

3) Es wird zu prüfen sein, ob nicht die von PAGE (1940) beschriebene erhöhte Fähigkeit des Hypertoniker-Serums, bei der Inkubierung mit Renin Hypertensin zu bilden, mit dem Depressan-Mangel dieser Kranken zusammenhängt.

4) HARRISON, GROLLMAN u. WILLIAMS (1940) sowie PAGE u. Mitarb. (1940, 1941) haben »Antipressorstoffe« aus Nieren beschrieben. Die bei Anwendung dieser Stoffe an Patienten beobachteten langdauernden Blutdrucksenkungen entsprechen vollkommen den Wirkungen, wie sie durch Depressan (= Vadil) erzielt wurden. Das gleiche gilt auch für die allgemeinen klinischen Resultate, insbesondere die Beeinflussung von hypertonischen Augenhintergrundsveränderungen, aber auch für die gelegentlichen unerwünschten Nebenwirkungen (Fieber usw.). Dabei wurde eine Einzeldosis des »Antipressorstoffes« aus 800 bis 1,000 g frischer Niere gewonnen, das für den gleichen Effekt notwendige Depressan aus 50 bis 80 ml Harn. Es wird zu prüfen sein, ob nicht die Wirkungen dieser Nierenpräparate nur auf ihrem Depressan-Gehalt beruhen.

Abschliessend ist festzustellen: Der am Anfang dieser Arbeit konstatierte scheinbare Antagonismus von Depressan und Renin

kommt nicht durch den gegensätzlichen Effekt auf das Erfolgsorgan zu Stande, sondern durch die Einwirkung des Depressans auf den enzymatischen Prozess, in dem das blutdrucksteigernde Hypertensin gebildet wird. Bei einer gegebenen Menge Renins und Serum-Globelins bzw. des in ihm enthaltenen Hypertensinogens hängt es von der Menge des gleichzeitig anwesenden Depressans ab, ob es zu der Bildung des blutdrucksteigernden und darmkontrahierenden Hypertensins kommen kann, oder ob dessen Bildung verhindert wird. Man wird das *in vitro* konstatierte Verhalten auch *in vivo* erwarten dürfen.

Zusammenfassung.

1) Das Hypertensinogen, der »Renin-Aktivator«, ist in der gleichen Pseudoglobulin-Fraktion des Serums enthalten, wie der »Inaktivator« des thermostabilen gefäßerweiternden Stoffes aus Harn, des Depressans.

2) Der Zusatz von Renin zu einem Inkubationsansatz von Depressan und Serum-Globulin hemmt die Inaktivierung von Depressan nicht. Dagegen verhindert der Zusatz von Depressan zu dem Inkubationsansatz von Renin und Serum-Globulin die Bildung von Hypertensin (= Angiotonin), was an der fehlenden Blutdruck- und Darmwirkung des Reaktionsproduktes demonstriert wird.

3) Die für diesen Effekt minimal notwendigen Mengen Depressan sind ungefähr die, die maximal von den gleichen Mengen Serum-Globulin unter den selben Bedingungen total inaktiviert werden.

4) Der thermolabile gefäßerweiternde Stoff aus Harn, Kallikrein, verhindert die Bildung von Hypertensin nicht.

5) Einige Konsequenzen dieser Einwirkung des Depressans auf die Hypertensinbildung werden diskutiert.

Literaturverzeichnis.

BRAUN-MENENDEZ, E., J. C. FASCIOLI, L. F. LELOIR, und J. M. MUÑOZ, Rev. Soc. Argent. Biol. 1939. *15*. 420.
 BRAUN-MENENDEZ, E., J. C. FASCIOLI, L. F. LELOIR, und J. M. MUÑOZ, J. Physiol. 1940. *98*. 283.
 EDMAN, P., U. S. v. EULER, E. JORPES, und T. SJÖSTRAND, Ebenda 1942. *101*. 284.

ELLIOT, A. H., und F. R. NUZUM, Endocrinology 1934. 18. 462.
EULER, U. S. v., Nord. Med. 1942. 13. 498.
EULER, U. S. v., und T. SJÖSTRAND, Acta physiol. scand. 1941. 2. 264.
274.
EULER, U. S. v., und T. SJÖSTRAND, Ebenda 1943. 5. 183.
FREY, E. K., und H. KRAUT, Hoppe-Seyl. Z. 1926. 157. 32.
FREY, E. K., und H. KRAUT, Münch. Med. Wschr. 1928. nr. 18. 763.
FREY, E. K., und H. KRAUT, Arch. exp. Path. Pharmak. 1928. 133. 1.
GROLLMAN, A., J. R. WILLIAMS jr., und T. R. HARRISON, J. biol. Chem. 1940. 134. 115.
HARRISON, T. R., A. GROLLMAN, und J. R. WILLIAMS jr., Amer. J. Physiol. 1940. 128. 716.
HARRISON, T. R., A. GROLLMAN, und J. R. WILLIAMS jr., J. Amer. med. Ass. 1940. 115. 1169.
KOHLSTAEDT, K. G., O. M. HELMER, und I. H. PAGE, Proc. Soc. exp. Biol., N. Y. 1938. 39. 214.
KOHLSTAEDT, K. G., I. H. PAGE, und O. M. HELMER, Heart 1940. 19. 92.
KRAUT, H., E. K. FREY, und E. BAUER, Hoppe-Seyl. 1928. 175. 97.
KRAUT, H., E. K. FREY, und E. WERLE, Ebenda, 1930. 189. 97. 192. 1.
KRAUT, H., E. K. FREY, E. WERLE, und F. SCHULTZ, Ebenda 1934. 230. 259.
MUÑOZ, J. M., E. BRAUN-MENENDEZ, J. C. FASCIOLI, und L. F. LELOIR, Nature 1939. 144. 980.
MUÑOZ, J. M., E. BRAUN-MENENDEZ, J. C. FASCIOLI, und L. F. LELOIR, Amer. J. med. Sci. 1940. 200. 608.
PAGE, I. H., J. exp. Med. 1939. 70. 521.
PAGE, I. H., Ebenda 1940. 72. 301.
PAGE, I. H., Amer. J. Physiol. 1940. 130. 29.
PAGE, I. H., Amer. Ass. Adv. Sci. 1940. nr. 13. 239.
PAGE, I. H., und O. M. HELMER, J. exp. Med. 1940. 71. 29.
PAGE, I. H., O. M. HELMER, K. G. KOHLSTAEDT, P. J. FOUTS, und G. F. KEMPF, Ebenda 1941. 73. 7.
TIGERSTEDT, R., und P. G. BERGMAN, Skand. Arch. Physiol. 1898. 8. 223.
WOLLHEIM, E., Schweiz. Med. Wschr. 1936. nr. 49. 1231.
WOLLHEIM, E., Acta med. scand. 1937. 91. 1.
WOLLHEIM, E., und K. LANGE, Dtsch. med. Wschr. 1932. 58. 572.

From the Chemistry Department of Karolinska institutet,
Stockholm.

A Micromethod for the Estimation of Phosphatidyl Ethanolamine in Nerve Tissue.

By

P. V. EDMAN and S. E. G. ÅQVIST.

Received 20 June 1945.

Investigations of the quantitative chemical composition of nervous material are considerably restricted because of lack of accurate methods for the estimation of lipids. This is especially the case with the cephalin fraction. Available methods include fractional extraction or precipitation of the lipids and calculation by difference for the estimation of this substance (KIRK 1938, WILLIAMS and coworkers 1938). Consequently these procedures are neither specific nor accurate. Furthermore, FOLCH (1942) has demonstrated the presence of two or perhaps three different "cephalins", one of which has the chemical composition formerly attributed to the whole cephalin by FOLCH termed phosphatidyl ethanolamine. The other one contains serine as the sole nitrogenous component, phosphatidyl serine. In addition there is an inositol containing cephalin fraction of not yet established composition.

The present authors have made an attempt to estimate micro quantities of phosphatidyl ethanolamine in a more specific way. This can be done by titration with acid of the ethanolamine distilled off from a hydrolysate of the lipids (BRANTE 1940). For the purpose of estimating smaller amounts of ethanolamine, however, we have found the Berthelot reaction (BERTHELOT 1859) with hypochlorite and phenol more suited because of its greater sensitivity. FÜRTH et. al. (1938) have studied this reaction more in

detail. It is produced by ammonia and certain primary amines, with a wide variety of colors. With ethanolamine in small quantities an intensely blue and stable color is obtained. By a certain procedure including distillation of the ethanolamine it is yet possible to eliminate other color developing substances.

Method.

The nerve tissue is finely minced and extracted with ethyl alcohol for 24 hours in a Soxhlet apparatus. The extract is diluted with alcohol to a suitable volume. 1 ml of this solution containing 0.4—1.5 mg of phosphatidyl ethanolamine is measured off into a centrifuge tube, 2 ml of 2 N hydrochloric acid is added and the tube is placed in a boiling water bath. Heating is continued for 3 hours during which time ethanolamine is quantitatively split off. After cooling, the tube is placed in a desiccator and the sample dried in vacuo at room temperature over solid sodium hydroxide. The fatty acids set free by the hydrolysis are extracted with 5 ml of dry ethyl ether. The undissolved residue is centrifuged down, the ether discarded, and the residue dissolved in 0.5 ml of distilled water. The sample is now ready for distillation which is performed in the apparatus shown in fig. 1.

It is transferred by suction to the distillation vessel. The sides of the tube are washed twice with 0.5 ml of water and once with 0.5 ml of ethyl alcohol. Then 0.5 ml of 1 N sodium hydroxide is added to the contents in the distillation vessel. The total volume would not exceed 3 ml. Ammonia is now removed by distilling in vacuo without receiver for 5 minutes at a temperature of 30—40° C. If the boiling is too violent it can be regulated by opening the lower stop-cock at L. The evaporation of ammonia is facilitated by the alcohol. No ethanolamine escapes during this procedure. Thereafter the receiver containing exactly 0.5 ml of 0.1 N hydrochloric acid is placed in position and its height adjusted so that the tip of the glass tube (G) dips a few millimeters under the surface of the hydrochloric acid. The contents in the distillation vessel are now distilled at a pressure of 10—15 mm Hg and a temperature of 80° C. in the heating bath to dryness and from that point the distillation is continued for another 5 minutes. Next, 1 ml of water is added to the distillation vessel from the cup (E) by tilting the apparatus slightly and carefully

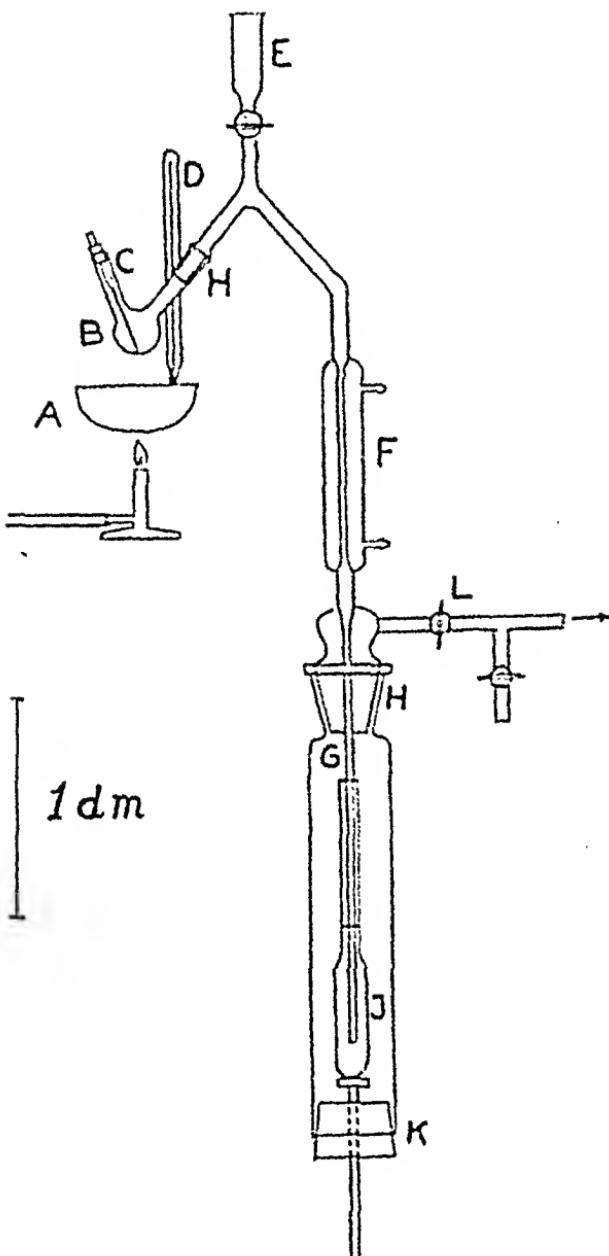


Fig. 1. Distillation apparatus. A is a heating bath containing Wood's metal; B, distillation vessel having a 10 ml volume; C, thin capillary; D, thermometer; E, cup with a stop-cock; F, condenser; G, glass tube 2 mm in bore; H, ground-in joint; J, receiver with a mark on the neck at the 5 ml volume; K, rubber stopper fitted with an appliance for adjusting the height of the receiver; L, stop-cocks. The distillation vessel should be made from Jena glass but the other parts can be made from ordinary glass.

opening the stop-cock. The distillation is repeated once again in the same way. The glass tube (G) is washed by tilting the apparatus and adding 0.5 ml of water from the cup (E). By this procedure ethanolamine is quantitatively, or practically so, transferred to the receiver.

The contents of the receiver are neutralized with 0.5 ml of 0.1 N sodium hydroxide and 0.5 ml of borate buffer is added. This addition will give a pH of 10 at which the color reaction has an optimal degree of sensitivity. Next, 0.5 ml of sodium hypochlorite is added, the contents diluted to the 5 ml-mark, mixed well by inverting, and allowed to stand for 15 minutes. Now 0.2 ml of phenol reagent is added and the contents mixed well. Blanks are made with water and the reagents. The tubes are placed in a vigorously boiling water bath for 5 minutes. A blue color is obtained which is stable for at least 6 hours. After cooling, the samples are ready for photometry in the Pulfrich step photometer. Filter S₆₁ and 1 cm cuvettes are used. The color development strictly follows the Lambert-Beer's law. We have found the extinction for 100 γ of ethanolamine to be 0.83, but it may vary somewhat, making the use of a standard necessary when high accuracy is desired.

Calculation.

By calculating the fraction of ethanolamine in phosphatidyl ethanolamine the assumption is made that the constituent fatty acids are stearic and oleic acid. Then the ethanolamine found should be multiplied with the factor 12.2 in order to obtain the content of phosphatidyl ethanolamine.

Reagents.

1. Ethyl ether, dried over calcium chloride.
2. Ethyl alcohol, 96 per cent.
3. Boric acid — sodium hydroxide buffer.

7.32 g boric acid (SÖRENSEN) and 2.00 g sodium hydroxide are dissolved in distilled water to 100 ml. This solution is supersaturated and must be warmed before use. When diluted 1:10 it will give pH 10.

4. Sodium hypochlorite.

To 0.15 N chlorine water 1 N sodium hydroxide is added to pH 10. This solution should be freshly made each time.

5. Phenol.

7.3 g phenol pro analysi is dissolved in 10 ml of distilled water. Strong sodium hydroxide is added to pH 10 and the solution diluted to 20 ml. This reagent is made afresh as soon as it has darkened.

6. Ethanolamine standard.

0.5 mg ethanolamine per ml of distilled water.

In order to test various points of the method a number of experiments were made. Samples of a solution of pure ethanolamine in water were distilled according to the directions given. An average recovery in the distillate of 97.5 per cent was obtained (Table 1).

Table 1.

Quantity of ethanolamine distilled	recovered	Percentage recovery
50 γ	49 γ	98
50 γ	48 γ	96
100 γ	97 γ	97
100 γ	96 γ	96
150 γ	148 γ	99
150 γ	148 γ	99
Average		97.5

To ensure that none of the ethanolamine was extracted by the ether under the conditions given, a solution containing ethanolamine and soaps of fatty acids in the ratio 1 : 10 was prepared. Aliquots of this solution were acidified with hydrochloric acid and treated as described above. The average recovery of ethanolamine was 97.5 per cent.

To the same solution ammonia was added so that the ratio of ethanolamine to ammonia was 1 : 1. Samples were treated as above. The average recovery of ethanolamine was 97.5 per cent showing that ammonia had been quantitatively eliminated.

Varying amounts of ethanolamine added to an alcoholic brain extract were quantitatively recovered.

Some analysis for phosphatidyl ethanolamine in grey and white matter of rabbit brain were made. Grey matter was obtained by carefully scraping off the cortex from the hemispheres and white matter was taken from the subcortical areas and corpus callosum. The percentage contents calculated on fresh tissue of phosphatidyl ethanolamine in grey and white matter were found to be respectively 0.81 and 0.27.

Summary.

A method is given for the quantitative estimation of phosphatidyl ethanolamine in nerve tissue by the content of ethanolamine. An alcoholic extract of the tissue is hydrolyzed, ethanolamine is distilled off and estimated colorimetrically by the Berthelot reaction.

The method is applied to 0.4—1.5 mg quantities of phosphatidyl ethanolamine.

Bibliography.

BERTHELOT, M., *Repertoire de chimie appliquée* 1859, page 284.
BRANTE, G., *Biochem. Z.* 1940. *305*. 136.
FOLCH, J., *J. biol. Chem.* 1942. *146*. 35.
FÜRTH, O., A. FRIEDRICH, and R. SCHOLL, *Biochem. Z.* 1938. *240*. 50.
KIRK, E., *J. biol. Chem.* 1938. *123*. 623.
WILLIAMS, H. H., B. N. ERICKSON, I. AVRIN, S. S. BERNSTEIN, and
I. G. MACY, *Ibidem* 1938. *123*. 111.

From the Biochemical Institute of the University, Aarhus,
Denmark.

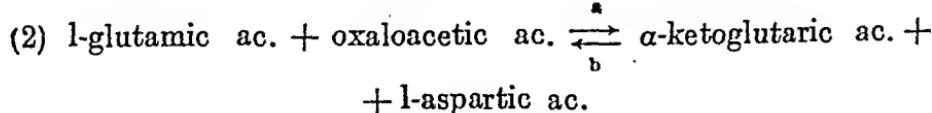
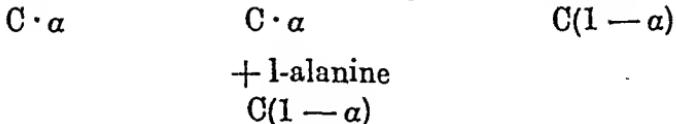
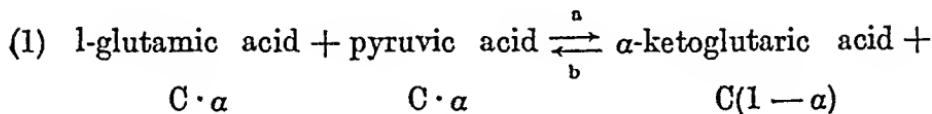
Kinetic and Thermodynamic Investigations on the Transamination Process.

By

SVEN DARLING.

Received 21 June 1945.

The transamination process has first been observed and described by BRAUNSTEIN and KRITZMANN (1937). Later BRAUNSTEIN (1940) has published a review on works by BRAUNSTEIN and collaborators, which have been reported mainly in Russian journals. From these works it appears that all amino acids with a few exceptions can react with α -ketoglutaric acid or oxaloacetic acid in the presence of an enzyme, transaminase, with the formation of L-glutamic acid and L-aspartic acid respectively, the amino acids being transformed into the corresponding keto acids. According to COHEN (1940 a, b) the possibility of transamination is, however, limited to the following two cases.



Without going into details with regard to the reason for the discrepancy between the results of these investigators, the author has limited the investigations to the kinetics of reactions 1 and 2, which have been stated with certainty.

In this work the apparent order of reaction, together with the equilibrium constant at different temperatures of the two reactions, catalyzed by a purified calf-heart transaminase, has been determined. On the basis of these values the activation energy E , the heat of reaction ΔH , change in free energy ΔF and Q_{10} are determined for reaction 1. At the same time the equilibrium constant of reaction 2 is determined. From these values ΔF of pyruvic acid and α -ketoglutaric acid are computed.

Experimental.

Analytical Procedures.

Amino Acids. Glutamic acid, aspartic acid and alanine are determined by a chromatographic method (DARLING, 1945).

Photometric Determination of Keto Acids. Pyruvic acid and oxaloacetic acid are determined photometrically by Straub's method, modified by DARLING (1944).

α -ketoglutaric acid also forms a yellow compound with salicylic aldehyde ($\epsilon_{PA}/\epsilon_{KGA} = 70$), hence all the pyruvic and oxaloacetic acid values will be found too high and a correction must be introduced. Instead of using an extinction curve determined by measuring a series of pure pyruvic acid solutions, a standard curve was obtained from measurements of the extinction of a series of solutions containing pyruvic and α -ketoglutaric acid, where $C_{PA} + C_{\alpha\text{-KGA}} = \text{constant}$ and the ratio $C_{PA}/C_{\alpha\text{-KGA}}$ varies in accordance with the stoichiometric equation. As C_{PA} and $C_{\alpha\text{-KGA}}$, under the given experimental conditions, vary between 12.5 and 0 micromols per ml and $C_{\alpha\text{-KGA}}$ between 0 and 12.5 micromols per ml in the solutions which are to be measured, a series of solutions are prepared by mixing 0.0125 M pyruvic acid and 0.0125 M α -ketoglutaric acid solutions. About 0.2 ml solution is pipetted off with a semi-automatic pipette, LEVY (1936), (with our pipette 0.196 ml). To this are added 0.80 ml water and the pyruvic acid reagents. The extinction is measured. An extinction curve is drawn by plotting 10^3E as ordinate against C_{PA} as abscissa. This curve is linear in the concentration range examined, and the intercept made on the ordinate axis by this line is $10^3E = 7$. From this curve are then obtained the true contents of pyruvic acid and oxaloacetic acid respectively corresponding to a given extinction of the reaction mixtures.

Titration of Keto Acids. If the keto acids are present separately or if the total amount in mixtures is to be determined the modified method of Fromageot and Desnuelles (DARLING, 1944) is used. When oxaloacetic acid is present this is first converted into pyruvic acid by heating to 70° for 160 minutes before the titration.

Substrate.

Amino Acids. L-glutamic acid (Merek) was purified after a method described in Organie Syntheses (1932). L-Alanine and L-aspartic acid (Hoffmann-La Roche) were perfectly pure.

Keto Acids. α -ketoglutaric acid was synthesized according to GLUTTERBUCK (1927), and oxaloacetic acid according to WOHL and ÖSTERLIN (1910). Pyruvic acid was used as sodium salt. This was obtained from Hoffmann-La Roche Ltd, to whom the author is very grateful.

Preparation of Enzyme.

T_0 . Calf-hearts, fresh or stored in a solid, frozen state, are hashed in a meat grinder and twice washed by suspending in 3 volumes of water. After the second washing the tissue is pressed almost dry through a linen cloth. The tissue is ground up with sand and extracted with 200 ml 1/15 M phosphate buffer, pH 7.15, for 2 hours at 35°. The tissue is pressed dry and the extract, which contains the enzyme, is centrifuged. The supernatant liquid is filtered through filter-paper. Nitrogen content: about 2.2 mg N per ml.

T_1 . 1 volume of T_0 is half-saturated by adding 1 volume of saturated ammonium sulfate. The precipitate which contains all the enzyme is filtered off, suspended in 25 to 50 ml water and dialyzed in a cellulose tube against tap water. When all ammonia has been removed $\frac{1}{3}$ volume of 0.2 M phosphate buffer, pH 7.15, is added. The insoluble residuum is centrifuged off and discarded. To the clear solution is added ethanol as antiseptic. When stored in refrigerator, it remains active for a week. Nitrogen content: about 0.80 mg N per ml. The enzyme preparations do not decarboxylize or oxidize the substrates, which is controlled by the above-mentioned analytical methods.

Determination of the Transaminase Activity.

The substrates are neutralized with a KOH-solution and made up with water and 0.2 M phosphate, pH 7.15, as 0.05 M solutions with regard to amino or keto acids and 1/15 M with regard to phosphate. 1 volume amino acid and 1 volume keto acid are mixed and placed in a water thermostat adjusted at 25.00° or 35.00° \pm 0.01°. When the mixture and the enzyme have assumed the temperature of the bath 1 volume of enzyme is pipetted into the flask of substrate. The mixture is shaken well and 3.00 ml are immediately withdrawn from the solution. The 3 ml are run into 1.00 ml 10 % trichloroacetic acid in order to arrest the reaction. The moment at which the solution is run into the trichloroacetic acid is noted; this is taken as the starting-point of the reaction.

After about 5 to 10 minutes 3.00 ml are withdrawn and the reaction is arrested. Further tests are withdrawn from time to time in the same manner at suitable intervals. The unit of time is second.

The degree of transamination, α' (Equation 1, left-right) is computed from the values of pyruvic acid concentration, by the formula:

$$(3) \quad \alpha' = \frac{C_{PA}^0 - C_{PA}^t}{C_{PA}^0}$$

where C_{PA}^0 is the initial concentration and C_{PA}^t is the concentration of pyruvic acid at the time t . Terms similar to this are deduced for the other components.

Results.

Kinetics of Reaction 1. In what follows some experiments are given, elucidating the order of the reaction of 1a and 1b. In Table I are given the results from one experiment (a) carried out at 35°.¹ The endpoint of the reaction is verified through 3 separate experiments (b, c, d) where the reaction mixture has been incubated for 24 hours. It is seen from the table that the reaction goes to an equilibrium, which is characterized by $\alpha' \rightarrow \alpha'_0$ and $\alpha'' \rightarrow \alpha''_0$. α'_0 is the degree of reaction from left to right and α''_0 the degree of reaction from right to left, when the equilibrium has been attained. α'_0 and α''_0 are linked together by the equation: $\alpha'_0 + \alpha''_0 = 1$. The α'_0 values given in the last column are computed from Equation 5 a. To examine whether the reaction is of 1. order k' and k'' , the unimolecular velocity constants for the processes 1a and 1b respectively, are computed from the equation

$$(4) \quad \ln(1-a) = 2.303 \log(1-a) = -kt$$

by inserting α' and α'' . The values are collected in Table I. It is seen that k decreases when the reaction proceeds. This is also to be expected, as the reaction goes to an equilibrium.

It was therefore tried whether the a -values satisfy the equation:

$$(5) \quad \ln\left(1 - \frac{a}{a_0}\right) = 2.303 \log\left(1 - \frac{a}{a_0}\right) = -\frac{a}{a_0}kt.$$

This equation holds for reciprocal processes, $A \xrightleftharpoons[b]{a} B$, where the state approaches an equilibrium through a reaction of 1. order. For 1a $a_0 = \alpha'_0 = 0.59$ and for 1b $a_0 = \alpha''_0 = 0.41$. In Fig. 1

¹ with a Ti-preparation.

Table I.

Determination of Velocity Constant and Equilibrium Constant for Reaction 1 at 35°.

Experiment	Substrate	L-Glutamic acid + pyruvic acid		L-Alanine + α -ketoglutaric acid		α_0' calculated
		Time sec.	α'	10 ⁴ k'	α''	10 ⁴ k''
a	0	0.000	—	0.000	—	—
	300	0.100	3.54	0.072	2.50	0.582
	600	0.180	3.32	0.125	2.24	0.591
	1200	0.275	3.52	0.190	1.76	0.593
	1800	0.350	2.47	0.240	1.53	0.599
	2700	0.414	1.98	0.294	1.29	0.584
	3600	0.400	1.87	0.310	1.03	0.613
	5400	0.563	1.53	0.390	0.91	0.591
	7200	0.581	1.21	0.395	0.70	0.585
	10000	0.595	0.88	0.420	0.55	0.584
	24 hours	0.590	—	0.410	—	0.592
b	24 hours	0.580	—	0.400	—	—
c	»	0.595	—	0.425	—	—
d	»	0.590	—	0.430	—	—
	Average	0.59	—	0.42	—	0.59

$\log \left(1 - \frac{\alpha}{\alpha_0}\right)$ is plotted against the time in sec. It is seen that the equilibrium point is reached through a 1. order reaction, and the

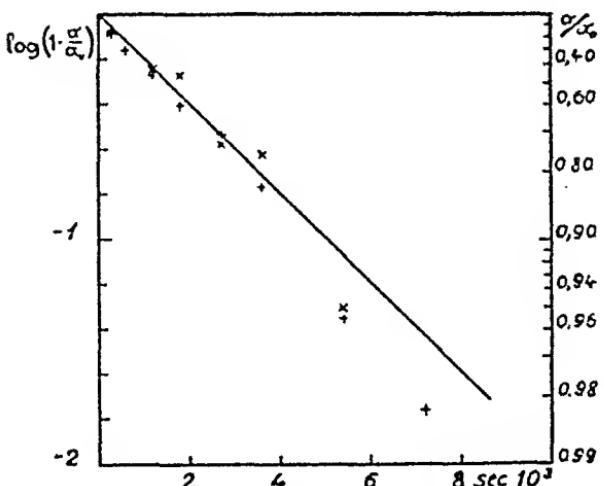


Fig. 1.

+ ————— + Reaction 1a
 x ————— x Reaction 1b

equation holds for a degree of reaction corresponding to 80 % of the final degree of reaction.

As Equation 5, according to its derivation, is valid for reaching the equilibrium, whether the reaction starts from right or left, one has got a means of evaluating α_0' . If the two opposite reactions are started at the same time, $1 - \frac{\alpha'}{\alpha_0'} = 1 - \frac{\alpha''}{\alpha_0''}$ at corresponding times. As $\alpha_0'' = 1 - \alpha_0'$ we obtain:

$$(5 \text{ a}) \quad \alpha_0' = \frac{\alpha'}{\alpha' + \alpha''} \text{ and } \alpha_0'' = \frac{\alpha''}{\alpha' + \alpha''}.$$

α_0' in the last column is computed from α' and α'' . The agreement between the calculated and directly determined values is very satisfying.

In a similar experiment with enzyme T_1 , α_0' for reaction 1 is determined at 25° on the basis of the change in the concentration of pyruvic acid. The results are collected in Table II.

Table II.
Determination of α_0' for Reaction 1 at 25° .

Time sec.	α'	α''	α_0'
0	0.000	0.000	—
900	0.155	0.117	0.570
1800	0.266	0.177	0.600
3600	0.367	0.250	0.595
6000	0.414	0.300	0.580
9000	0.468	0.325	0.590
24 hours	0.570	0.380	0.600
		Average	0.59

It is seen that the change in temperature apparently is without influence on the equilibrium.

In order to demonstrate the agreement between the values of α' , (determined by the change in glutamic acid and pyruvic acid concentration) for reaction 1 with a T_1 -preparation, the values of α' inserted in $\log \left(1 - \frac{\alpha'}{0.59} \right)$ are plotted against the time in Fig. 2.

It is seen from the figure that the two sets of points determine the same straight line. This is also to be expected when no side-reactions occur.

Determination of Q_{10} for Reaction 1. To determine the temperature coefficient, Q_{10} , of reaction 1, the velocity constants k_{25} and k_{35} with crude and purified enzymes are measured. As substrates serve glutamic acid and pyruvic acid. The change in the pyruvic acid concentration is reckoned from the beginning of the reaction. The velocity constant is calculated in accordance with Equation 5. The temperature coefficient of the reaction is calculated from: $Q_{10} = \frac{k_{35}}{k_{25}}$. The values are given in Tables III and IV. The deviation between the two values is of the same order of magnitude as the experimental error. On an average $Q_{10} = 2.29$.

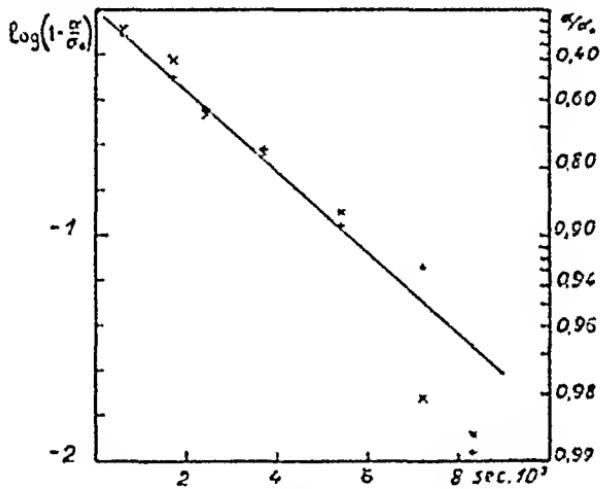


Fig. 2.

+ —————+ Determined by change in pyruvic acid
 × —————× Determined by change in glutamic acid.

Calculation of the Equilibrium Constant of Reaction 1. According to the law of mass action, the following equation is deduced for the equilibrium constant:

$$(6) \quad K = \left(\frac{\alpha'_0}{1 - \alpha'_0} \right)^2 = \left(\frac{1 - \alpha''_0}{\alpha''_0} \right)^2$$

where the symbols have the above-mentioned signification. As α'_0 at 35° is equal to α'_0 at 25° we obtain. $K_{35} = K_{25} = 2.07$, by inserting $\alpha'_0 = 0.59$ in Equation 6. It must be emphasized, that K is not the thermodynamic constant, and thus the condition of equilibrium is not given correctly. An attempt to calculate the true constant is, however, omitted on account of the insufficiency of data regarding the activity coefficients of the reactions.

Table III.

Determination of Q_{10} for T_0 .

$t = 25^\circ; \alpha'_0 = 0.59$			$t = 35^\circ; \alpha'_0 = 0.59$		
Time sec.	α'	$10^4 k_{25}$	Time sec.	α'	$10^4 k_{35}$
0	0.000	—	0	0.000	—
660	0.072	(1.96)	600	0.288	11.2
1200	0.204	3.54	1200	0.407	9.75
1920	0.374	5.22	1800	0.479	9.30
2700	0.387	3.96	2400	0.521	8.99
	Average	4.24		Average	9.80
$Q_{10} = 2.31$					

Table IV.

Determination of Q_{10} for T_1 .

$t = 25^\circ; \alpha'_0 = 0.59$			$t = 35^\circ; \alpha'_0 = 0.59$		
Time sec.	α'	$10^4 k_{25}$	Time sec.	α'	$10^4 k_{35}$
0	0.000	—	0	0.000	—
600	0.177	5.95	600	0.297	11.7
1200	0.261	4.88	1200	0.450	12.3
1800	0.363	5.30	1800	0.528	12.5
	Average	5.38		Average	12.2
$Q_{10} = 2.27$					

The Equilibrium Constant of Reaction 2. The degree of reaction in the equilibrium, α'_0 , is determined by measuring the corresponding values of α'_0 and α'' at different times. The determination is carried out at 25° with a T_1 -preparation. The calculations are based on the change in oxaloacetic acid and the values are given in Table V. The equilibrium constant is computed from Equation 6 by inserting α'_0 . This gives $K_{25} = 5.44$. The comments, which apply to the equilibrium constant of reaction 1 are valid here too.

Table V.

Determination of α'_0 for Reaction 2 at 25°.

Time sec.	α'	α''	α'_0
0	0.000	0.000	—
300	0.280	0.112	0.715
600	0.434	0.186	0.700
1200	0.546	0.268	0.670
1800	0.620	0.266	0.700
		Average	0.70

Thermodynamics of Reaction 1.

From the experimental data it is now possible to calculate some important thermodynamic constants for the transamination process catalyzed by enzyme from calf-heart.

The Energy of Activation, E. The effect of temperature on the rate of reaction is represented by the Arrhenius equation, integrated between T_1 and T_2 .

$$(7) \quad E = \frac{RT_1T_2}{T_2 - T_1} \ln \frac{k_2}{k_1}$$

where $R = 1.987$ cal. per degree, k_2 and k_1 are the velocity constants at the absolute temperatures T_2 and T_1 . When k_2 and k_1 are measured at 35° and 25° C we have $k_2/k_1 = Q_{10}$. By inserting the average value, $Q_{10} = 2.29$, $T_2 = 308^\circ$ and $T_1 = 298^\circ$ K we obtain:

$$E = \frac{1.987 \times 298 \times 308}{308 - 298} \times 0.8286 = 15,110 \text{ cal.}$$

The Heat of Reaction, ΔH . When the equilibrium constants K_1 and K_2 at the temperatures T_1 and T_2 are known, we obtain ΔH from the equation:

$$(8) \quad \Delta H = \frac{RT_1T_2}{T_2 - T_1} \ln \frac{K_2}{K_1}$$

As $K_{35} = K_{25} = 2.07$ we obtain: $\Delta H = 0$.

The Change in Free Energy, ΔF . ΔF represents the maximum of useful work obtainable from the process at constant temperature and pressure. ΔF is calculated by the relation:

$$(9) \quad \Delta F = -RT \ln K,$$

where K is the equilibrium constant. As $K_{35} = K_{25}$ of reaction 1, we obtain by inserting $K_{25} = 0.59$, $T_1 = 298$ and $T_2 = 308$: $\Delta F = -0.43$ kcal at $25^\circ C$ and $\Delta F = -0.45$ kcal at $35^\circ C$ of reaction 1. By inserting $K_{25} = 5.44$ we obtain: $\Delta F = -1.00$ kcal at $25^\circ C$ of reaction 2.

Calculation of ΔF of Pyruvic Acid and α -Ketoglutaric Acid. ΔF of the reactions 1 and 2 are also given by the equations:

$$(10) \quad \Delta F = \Delta F_{\text{KGA}} + \Delta F_A - \Delta F_{\text{GA}} - \Delta F_{\text{PA}}$$

$$(11) \quad \Delta F = \Delta F_{\text{KGA}} + \Delta F_{\text{AA}} - \Delta F_{\text{GA}} - \Delta F_{\text{OAA}}$$

As the experiments are carried out at pH 7.15, the aminodiacidic acids are found as monovalent anions, alanine in neutral form and the keto acids as divalent and monovalent anions. BORSOOK and HUFFMANN (1933) have determined ΔF of l-glutamic acid, l-aspartic acid and l-alanine; LEHMANN and HOFF-JØRGENSEN (1939) ΔF of oxaloacetic acid. The values are given in Table VI. By inserting in Equations 10 and 11 ΔF_{PA} and ΔF_{KGA} are obtained. The values are entered in Table VI.

Table VI.

Substance	Change in free energy
l-Aspartic acid monovalent anion (1)	$\Delta F_{\text{AA}}^\pm = -166.40$ kcal (25°)
l-glutamic acid monovalent anion (1)	$\Delta F_{\text{GA}}^\pm = -162.85$ *
l-Alanine neutral (1)	$\Delta F_A^\pm = -89.20$ *
Oxaloacetic acid divalent anion (2)	$\Delta F_{\text{OAA}}^- = -186.3$ *
α -Ketoglutaric acid divalent anion	$\Delta F_{\text{KGA}}^- = -183.8$ *
Pyruvic acid monovalent anion	$\Delta F_{\text{PA}}^- = -110.5$ kcal (25°)

It is seen, that the reaction investigated does not deviate materially from other enzymatic reactions with regard to the thermodynamic conditions. As ΔF is negative the process has a

1) BORSOOK & HUFFMANN (1933); 2) LEHMANN & HOFF-JØRGENSEN (1939).

tendency to take place spontaneously from left to right. The rate, however, is determined by the enzyme, and without enzyme it is impossible, in the investigated range of temperature to detect any change in the reaction mixture.

As a control of the probability of $\Delta F_{PA} = 110.5$ kcal serves the value $\Delta F_{PA} = 115$ kcal, judged by FRANKE (1933) from thermic data. The agreement is satisfying, as the heat of solution and dissociation are not considered in the judged value.

Summary.

By means of transaminase prepared from calf-heart, the reaction: l-glutamic acid + pyruvic acid \rightleftharpoons α -ketoglutaric acid + l-alanine is investigated.

1. The process reaches an equilibrium through a first-order reaction according to the equation:

$$\ln(1 - a/a_0) = -kt.$$

2. The equilibrium constant is:

$$K_{35} = K_{25} = 2.07.$$

3. The temperature coefficient of the reaction is:

$$Q_{10} = 2.29.$$

On the basis of these values, some thermodynamic constants of the reaction are computed.

4. The energy of activation is determined from Q_{10} .

$$E = 15.110 \text{ kcal.}$$

5. The heat of reaction calculated from K_{35} and K_{25} is:

$$\Delta H = 0 \text{ kcal.}$$

6. The change in free energy is:

$$\Delta F = -0.43 \text{ kcal (25°)}$$

$$\Delta F = -0.45 \text{ kcal (35°)}.$$

7. The free energy of the divalent α -ketoglutaric acid ion and the monovalent pyruvic acid ion are calculated:

$$\Delta F_{KGA} = -183.8 \text{ kcal (25°)}$$

$$\Delta F_{PA} = -110.5 \text{ kcal (25°)}.$$

This work has been aided by a grant from the "Kong Christian X's Fond".

References.

BORSOOK, H. and H. M. HUFFMANN, J. Biol. Chem. 1933. 99. 663.
BRAUNSTEIN, A. E., Enzymologia 1937. 2. 129.
BRAUNSTEIN, A. E. and M. G. KRITZMANN, Ibidem 1939. 7. 25.
COHEN, P. P., J. Biol. Chem. 1940 a. 136. 565.
COHEN, P. P., Ibidem 1940 b. 136. 585.
DARLING, S., Acta physiol. Scand. 1944. 7. 306.
DARLING, S., Ibidem 1945 in press.
FRANKE, W., Biochem. Z. 1933. 258. 280.
GLUTTERBUCK, P. W. Ibidem 1927, 21. 519.
LEHMANN, J. and E. HOFF-JØRGENSEN, Skand. Arch. Physiol. 1939. 82. 113.
LEVY, M., C. R. Lab. Carlsberg 1935. 21. 102.
KING, H., Organic Synthese collective Volume 1932. 1. 281.
WOHL, A. and C. ÜSTERLIN, Ber. dtsch. chem. Ges. 1910. 34. 1139.

(From the Departments of Biochemistry and Anatomy and Division of Dental Research, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)

Peroxidation of Body Fat in Vitamin E Deficiency.

By

HENRIK DAM and HUMBERTO GRANADOS.

Received 27 June 1945.

Certain symptoms of vitamin E deficiency are dependent upon or considerably accelerated by the ingestion of fat containing highly unsaturated fatty acids (DAM 1944 a and b). It has been suggested that these symptoms might be related to an abnormal oxidation of such acids in the tissues (DAM 1944 b). Indications of a disorder arising from this fundamental error in metabolism may be contained in the exudative diathesis and encephalomalacia in chicks (DAM 1944 a and b), in the brown discoloration of adipose tissue in rats and chicks, as well as in the depigmentation of the rat incisor (GRANADOS and DAM 1945). The pathological changes encountered in exudative diathesis and the brown pigmentation of adipose tissue particularly offer a convenient route for investigation of this problem inasmuch as these abnormalities involve readily accessible adipose tissue. In the present report, a study has been made of the peroxide values of adipose tissue from both chicks and rats under different conditions of vitamin E deficiency.

Experimental:

Peroxide determinations were made by the method of KING, ROSCHEN and IRWING (1933) modified for use with relatively small quantities of animal tissues. One half to 1 g of tissue was ground with 1½ times its weight of sodium sulfate and 4 to 8 ml of chloroform (based upon the weight of tissue taken), centrifuged, and filtered. The lipid content of the chloroform extract was determined by evaporating 0.5 to 1 ml

of the solution on a watch glass. Then a volume of chloroform solution corresponding to about 50 mg of lipid was transferred to a 25 ml Erlenmeyer flask. Chloroform was added to make a total of 1.2 ml of solution. To this was added 1.8 ml glacial acetic acid and 0.05 ml saturated aqueous potassium iodide solution (iodate free, freshly dissolved). After mixing, the solution was permitted to stand for 1 minute, then 5 ml of water and 1 drop of 1 per cent starch in saturated sodium chloride solution was added, the flask was swirled thoroughly and the liberated iodine was titrated with freshly prepared $\frac{N}{500}$ sodium thiosulfate. By freshly redistilling the chloroform and water used in the analyses, the blank value for the reagents was always zero. Peroxide values were calculated in milliequivalents per 1000 g of fat:

$$\text{peroxide value} = \frac{\text{ml } \frac{N}{500} \text{Na}_2\text{S}_2\text{O}_3 \times 2}{\text{g fat in sample}}$$

The limit of accuracy is about 0.5 unit.

The diets used in these tests were selected so as to produce the desired symptoms within reasonable time limits. The chicks were reared, from hatching, on our vitamin E deficient diet 182¹ (DAM 1944 a). The 5 per cent cod liver oil contained in this ration was added each day before feeding. The control chicks received either the same diet plus 2.5 mg d,l-alpha-tocopherol acetate (Ephynal Acetate, Roche) or a commercial ration.² Another group received the same amount of gamma-tocopherol acetate.³

The animals were sacrificed at different ages beginning with the onset of symptoms in the vitamin E deficient group. Samples of adipose tissue were taken for analysis from the fatty tissue on the left of the crop where initial deficiency is usually evidenced.

A study of the local peroxidation of injected cod liver oil in the presence or absence of vitamin E was also undertaken. Two week old chicks receiving the normal commercial diet were injected with 0.3 ml of cod liver oil subcutaneously in the pectoral region. A second group of similar chicks were given the same treatment plus 2.4 mg of the natural alpha tocopherol⁴ dissolved in the cod liver oil. A sample of the subcutaneous tissue from this region containing unabsorbed oil was taken when the animals were sacrificed.

The rats were reared on a similar vitamin E deficient diet except that the cod liver oil content was increased to 20 per cent while the sucrose content was correspondingly reduced. Control rats were given either the same diet plus 10 mg per cent of d,l-alpha-tocopherol acetate or the vitamin E deficient diet without cod liver oil. Samples

¹ Consisting of Casein, alcohol-extracted, 15 g; Dried yeast, ether-extracted, 10 g; Sucrose 54.6 g; Gelatine 8 g; Gum arabic 5 g; Salt mixture 7.2 g. (McCollum 185 supplemented with 1 mg KI, 10 mg CuSO₄, 5H₂O and 40 mg MnSO₄, 4H₂O in 7.2 g of the salts); Choline chloride 0.1 g; Cystine 0.1 g; cod liver oil 5 g; vitamin K substitute 1 mg.

² "Starting and growing mash" from Newman Bros. Grain Co., Rochester, N. Y.

³ Obtained from Distillation Products, Inc., Rochester, N. Y.

⁴ Obtained from Distillation Products, Inc., Rochester, N. Y.

of adipose tissue for analysis were taken from the subcutaneous, intraperitoneal and paraepididymal (pampiniform plexus) regions. The determinations were usually performed on mixed samples obtained from one animal. In some tests the subcutaneous and intraperitoneal fat was examined separately.

Results and discussion.

The data have been highly condensed in tabular form.

Chicks: Table 1 indicates that the adipose tissue of chicks fed on vitamin E deficient diet containing 5 per cent of cod liver oil

Table 1.¹

Chicks receiving the vitamin E deficient diet 182 containing 5 per cent of cod liver oil, supplemented as indicated.

d,l-alpha-tocopherol acetate mg %	Gamma-tocopherol acetate mg %	Days on diet	Per-oxide value	Changes of the adipose tissue			Encephalo-malacia
				hemorrhage	exudate	brown discoloration	
0	0	16	3.3	+++	+++	0	0
0	0	20	1.5	++	++	0	0
0	0	21	0	0	0	0	0
0	0	21	11.0	++	++	0	0
0	0	23	0	+++	+	0	0
0	0	27	0	0	0	0	0
0	0	30	0	0	0	0	0
0	0	30	35.2	(++)	(++)	++	0
0	0	31	0	0	0	0	0
0	0	34	0	0	0	0	0
0	0	34	15.7	(+)	0	++	+
0	0	35	0	(++)	(++)	++	0
0	0	35	0	0	0	+	0
0	0	35	2.5	(++)	(++)	+	0
0	0	36	0	(++)	(++)	+	0
0	0	36	38.7	(++)	(++)	++	+
0	0	41	25.6	(++)	(++)	+	0
0	0	41	13.2	(+)	(+)	+	0
0	0	42	39.3	(++)	(++)	+++	0
0	0	42	1.5	0	0	0	0
0	0	42	39.7	(++)	(++)	++	0
2.5	0	19	0	0	0	0	0
2.5	0	29	0	0	0	0	0
2.5	0	31	0	0	0	0	0
2.5	0	42	0	0	0	0	0
2.5	0	49	0	0	0	0	0
0	2.5	17	0.2	+++	+++	0	0
0	2.5	21	4.3	+++	++	0	0
0	2.5	40	20.3	(++)	(++)	0	0
0	2.5	42	0.5	(+)	0	++	0
0	2.5	49	0	0	0	0	0

¹ 0 indicates absence of symptom, + = light symptoms, ++ = marked symptoms, +++ = very marked symptoms.

Plusses in parenthesis indicate that the symptoms occurred but disappeared before the animal was sacrificed.

may contain peroxides. In several samples, the peroxide value exceeded the level which is considered the limit for rancidity of industrial fats, viz. 20 milliequivalents per 1000 g. of fat (KING, ROSCHEN and IRWIN 1933). When the diet contained 2.5 mg per cent d,l-alpha tocopherol acetate, the depot fat did not contain peroxides. This was observed in spite of the fact that the cod liver oil used in all the experiments had a peroxide value of 16. Therefore, it would appear that the peroxides found in the depot fat of the vitamin E deficient chicks must have originated in the tissue and could not result from an accumulation of the peroxides contained in the dietary fat. The depot fat of chicks reared on the commercial ration contained no peroxides. The appearance of peroxides in the depot fat must be a sign that this tissue is depleted of vitamin E and contains fatty acid of a type which is easily peroxidized in absence of antioxidants. The time required for the development of these conditions is subject to considerable individual variation. The present trials do not in themselves serve to clarify the relationship of peroxides to the symptoms occurring in the adipose tissue. Exudation and its initial stage, diffuse hemorrhage, have occurred while the peroxide content of the fat was very low or not demonstrable at all by the methods¹ available to us at the present time. Diffuse hemorrhage and exudation may disappear as the animals become older, in spite of the fact that the peroxide values are relatively high. The brown discoloration of adipose tissue which is usually observed subsequent to the aforementioned symptoms is more frequently found in the period when the peroxide values are high, although in a few cases even this symptom was not correlated with demonstrable peroxide values. If, therefore, there is a causative relationship between the peroxide formation and the symptoms, it seems that the adipose tissue may undergo changes in the course of which previously formed peroxides can no longer be detected within the limit of the method. The relationship between the peroxidation and the symptoms might be further clarified by similar trials using a less peroxidizable fat such as lard.

We were unable to detect any significant protection against peroxidation and symptoms with gamma-tocopherol acetate at a level of 2.5 mg per cent. Since it has been shown that gamma-

¹ In a previous study (DAM 1944 a) the author reported that there was no rancidity of the body fat concurrent with the onset of the symptoms, but the method used in that study was less sensitive than the technique described above.

Table 2.

Chicks receiving normal commercial diet, injected subcutaneously with 0.3 ml of cod liver oil with or without free natural tocopherols.

Tocopherol in injected oil, mg	Days after injection	Peroxide value	Color of sample
0	0	16	faintly yellow
0	3	38	light yellow
0	4	34	light yellow
0	5	199	yellow
0	5	272	yellow
0	6	260	yellow brown
0	7	181	yellow brown
0	8	99	yellow
0	10	84	yellow brown
0	15	68	yellow brown
0	20	33	yellow brown
0	26	9	brown
2.4	4	115	light yellow
2.4	5	205	yellow
2.4	6	199	yellow
2.4	7	372	light yellow
2.4	8	212	yellow

tocopherol is a more effective antioxidant *in vitro* than alpha-tocopherol (OLCOTT and EMERSON, 1937) these results suggest that gamma-tocopherol is deposited in the tissues to a lesser extent than alpha-tocopherol.

In the experiments where cod liver oil was injected into the subcutaneous tissue of chicks receiving a normal diet (table 2) a very considerable peroxidation took place. The peroxide values of samples from these animals far exceeded those found in the adipose tissue of vitamin E deficient chicks fed cod liver oil. No exudation occurred at the site of injection, an observation which could be interpreted as evidence against a causal relationship between peroxidation and exudation. However, it is pertinent to indicate that the peroxidation of a local deposit of cod liver oil might not necessarily affect the capillaries in the same manner as peroxidation occurring within the cells of the tissue as a consequence of a general deposition of an orally ingested highly unsaturated fat free of antioxidant. The development of very high peroxide values after the injection of cod liver oil even when the oil contained free natural alpha-tocopherol suggests that either the tocopherol is destroyed by the peroxidation of the oil (possessing a peroxide value of 16 before the injection), or has rapidly passed into the circulation.

Table 3.

Rats receiving vitamin E deficient diets with or without cod liver oil or similar diets supplemented with vitamin E.

Cod liver oil %	d,l-alpha-toco-pherol acetate mg %	Sex	Duration of feeding in days	Per-oxide value	Discoloration of adipose tissue			Discoloration of uterus
					subcutaneous	intraperitoneal	paraepididymal	
from weaning								
20	0	m	24	0	0	0	0	
20	0	f	24	0	0	0	0	0
20	0	f	26	0	0	0	0	0
20	0	m	26	0	0	0	0	0
20	0	f	31	3.6	0	0	0	0
20	0	m	31	11.4	0	0	+	
20	0	m	40	3.1	0	0	+	
20	0	f	51	4.3	0	+	+	0
20	0	f	65	5.0	0	++	++	0
20	0	m	115	9.9	+	+++	+++	
20	0	m	124	71	++	+++	+++	
20	0	m	152	72 ¹	++	+++	+++	
20	0	f	238	61	++	+++	+++	0
20	0	f	265	41	++	+++	+++	
20	0	f	314	33	++	+++	+++	++
from birth								
20	0	f	7	4.6	0	0	0	
20	0	f	15	4.9	0	0	0	
20	0	f ²	21	108	+++	+++	+++	
20	0	f	21	0	0	0	0	
20	0	m	35	17	++	++	++	
20	0	f	40	189	+++	+++	+++	++
20	0	f	51	128	+++	+++	+++	
from weaning								
0	0	f	231	0	0	0	0	+
0	0	f	259	0	0	0	0	0
0	0	f	308	0	0	0	0	+
20	10	f	237	0	0	0	0	0
20	10	f	265	0	0	0	0	0
20	10	f	314	0	0	0	0	0

¹ intraperitoneal fat; the subcutaneous fat had a peroxide value of 5.4.

² This animal died with very severe anemia and lipemia.

The injection of cod liver oil gave rise to the formation of yellow to brown fibrinous tissue in which the oil was imbedded, such as has been described by ENDICOTT (1944). The intensity of the color continued to increase even after the period during which the highest peroxide values were found.

Rats: When rats were fed the vitamin E deficient, high cod liver oil diet from weaning (table 3), peroxide values were found

in the fat from different parts of the body. Brown coloration was first observed in the pampiniform plexus (paraepididymal fat) 26 days after the beginning of the feeding, then in the intraperitoneal fat and still later in the subcutaneous fat. The discoloration of the uterus occurred much later. When the vitamin E deficient, high cod liver oil diet was given from birth, peroxide values and pigmentation of adipose tissue was observed earlier than seen when weanling rats were tested. The fact that paraepididymal fat is the first to be affected recalls the early testicular manifestations of vitamin E deficiency in male rats. On the other hand, the later appearance of discoloration of the uterus as compared with that of the adipose tissue, could be interpreted as meaning that the uterus discoloration is unrelated to dietary fat. The visible coloration of the adipose tissue was more uniformly preceded by peroxidation in rats than in chicks. This fact could be related to the difference in the amount of cod liver oil given in the two experiments. Histological examinations now in progress promise to reveal the presence of pigment before the latter can be observed grossly, which would suggest that the peroxides are related with the very formation of the pigment.

Visible pigment did not develop in the adipose tissue when the diet contained no fat or when vitamin E was present, a fact which is in agreement with the original observations on the pigmentation of adipose tissue (DAM 1944 a) and the disappearance of the enamel pigment of the rat incisor (GRANADOS and DAM 1945).

The following preliminary information is available as to the chemical characterization of the pigment which indicates that two separate components are involved. When the pigmented adipose tissue is ground with sodium sulfate and extracted with fat solvents some orange-brown colour is dissolved, but most of the brown coloring matter is unextractable with such solvents. This is also true when the tissue is extracted repeatedly with acetone and then with other fat solvents. Water or alcohol do not remove the pigment from the defatted tissue, but dilute aqueous alkali such as 1 per cent sodium hydroxide will slowly dissolve the pigment. The alkaline solution of the pigment shows a greenish-yellow fluorescence when viewed under the ultraviolet lamp. The pigment can be precipitated from its alkaline solution together with protein decomposition products by acidification or by addition of a large amount of acetone. Ether does not extract

Table 4.
Hemoglobin values of rats.

Diet	Days of experiment from birth	Hemoglobin %
Vitamin E deficient plus 20 % cod liver oil	22	10.7
	26	10.2
	35	16.8
	40	8.4
	51	8.2
Vitamin E deficient plus 10 mg % d,l-alpha-tocopheral acetate plus 20 % cod liver oil	22	9.7
	26	11.3
	35	8.5
	51	8.2
Normal stock diet (dog chow)	22	16.8
	26	16.2
	35	15.8
	51	16.8

the pigment from its alkaline solution or from the precipitate produced by acidification, even if the alkaline solution has been heated to 100° C for one hour before acidification. The pigment of the defatted tissue does, therefore, not seem to be a lipoid. Sodium dithionite does not bleach the alkaline solution of the pigment, thus excluding the possibility that it is identical with diaphorase. The ARNOW test (1937) shows that the pigment is not identical with melanin. Preliminary tests indicate further that the iron content of the pigmented and unpigmented adipose tissue is of the same order of magnitude and that there is no difference in the content of succinic dehydrogenase, whereas the catalase activity seems to be somewhat higher in the pigmented tissue. In sum, the characterization so far given agrees with the description of "Ceroid", the pigment found in various tissues in experimental liver cirrhosis in rats, caused by the feeding of diets low in choline (ENDICOTT and LILLIE 1944, GYORGY and GOLDBLATT 1942).

Furthermore, it is very likely that the brownish globules of pigment revealed in the histological study of the brown adipose tissue (DAM and MASON 1945) are similar to those found in other organs in vitamin E deficiency (MASON and EMMEL 1944 and 1945). This assumption is based upon the similar acid-fast and other staining reactions and properties which characterize both the pigment of the adipose tissue and that of the other organs.

ENDICOTT (1944) has considered the possibility that "ceroid" may be related to the oxidation of fat; the present observations show that such an oxidation does occur in the tissue studied.

The hemoglobin content of blood from rats fed 20 per cent cod liver oil diets from birth, with and without vitamin E (table 4), was found to be lower than in rats of the same age fed normal stock diet. This is in agreement with the observations of DAVIS (1944) in which a reduction in the hemoglobin level was obtained by feeding diets high in fat. The present data indicate that vitamin E has no influence, *per se*, on the anemia produced by high fat diets.

Summary.

1. Chicks fed vitamin E deficient diets containing 5 per cent cod liver oil developed peroxides in the adipose tissue during the period in which the known symptoms of exudation, and particularly brown coloration of the same tissue occurred. The relation between the peroxidation and the symptoms is discussed.

2. Subcutaneous injection of 0.3 ml of cod liver oil with a peroxide value of 16 to chicks receiving a normal diet, resulted in very marked peroxidation at the site of injection accompanied by a progressive deposition of yellow to brown fibrous material. This local peroxidation was not prevented by the incorporation of 2.4 mg of free natural alpha-tocopherols in the injected oil.

3. Rats reared on vitamin E deficient diets containing 20 per cent cod liver oil from weaning developed peroxides in the adipose tissue preceding the visible yellow-brown coloration which appeared first in the paraepididymal fat, then in the intraperitoneal, and finally in the subcutaneous fat. When females together with their offspring were given the same diet from the time of delivery, the symptoms in the young developed earlier than in weanling rats and were more marked.

4. The yellow-brown color of the adipose tissue is due to two substances, one of which is extractable with fat solvents, while the other, which occurs in larger quantity, is soluble in dilute alkali. A further, preliminary, characterization of this latter substance is given.

5. Vitamin E has no influence on the lowering of hemoglobin caused by feeding high cod liver oil diets to young rats.

Acknowledgement: This work was aided by grants from Wyeth Incorporated of Philadelphia and Eastman Dental Dispensary of Rochester.

References.

ARNOW, L. E., J. biol. Chem. 1937. *118*. 531.
DAM, H., J. Nutrit. 1944 a. *27*. 193.
DAM, H., Ibidem 1944 b. *28*. 297.
DAM, H. and K. E. MASON, Federation Proc. 1945. *4*. 153.
DAVIS, J. E., Amer. J. Physiol. 1944. *142*. 213.
ENDICOTT, K. M., Arch. Pathol. 1944. *37*. 49.
ENDICOTT, K. M., and R. D. LILLIE, Amer. J. Pathol. 1944. *20*. 149.
GRANADOS, H., and H. DAM, Science 1945. *101*. 250.
GYORGY, P., and H. GOLDBLATT, J. exp. Med. 1942. *75*. 355.
KING, A. E., H. L. ROSCHEN, and W. H. IRWIN, Oil and Soap 1933.
10. 105.
MASON, K. E. and A. EMMEL, Yale J. Biol. Med. 1944. *17*. 189.
MASON, K. E. and A. EMMEL, Anat. Rec. 1945. *92*. 33.
OLCOTT, H. S., and EMERSON, J. Amer. chem. Soc. 1937. *59*. 1008.

From the Medical Department A of the Rigshospital, Copenhagen.

Spectral Absorption of Bilirubin.

Measurements in Pure Aqueous Solutions and in Solutions Containing Human Serum.

By

TORBEN K. WITH.¹

Received 9 July 1945.

As the spectral absorption of bilirubin is deficiently elucidated new measurements are needed.

In the following all extinctions are given as $E_{1\text{ cm}}^{1\%}$ (extinction of a solution containing 1 g. per 100 ml. in a 1 cm. thick layer). In the literature most often the absorption constant (A) is given and from its definition (*cf.* HEILMEYER, 1933) the relation $E_{1\text{ cm}}^{1\%} =$

$= \frac{1}{100 A}$ is calculated. We shall now give values for $E_{1\text{ cm}}^{1\%}$ as calculated from the data of previous investigators.

YLPPÖ (1913; p. 231) gives an absorption curve for bilirubin in the region 580—480 m μ (*i. e.*, not including the maximum) from which the values 208 at 493 m μ and 36 at 538 m μ can be calculated for $E_{1\text{ cm}}^{1\%}$.

SHEARD *et al.* (1926) performed spectrophotometrical measurements on dilutions of dog bile but not on pure bilirubin solutions in the region 430—585 m μ . They found the greatest extinction at 430 m μ but whether this wave length corresponded to a maximum could not be seen as measurements were only carried out in the visible part of the spectrum. From comparison of their spectral measurements of the yellow color of bilirubin which were performed with an elaborate technique with measurements of the diazo reaction with the primitive colorimetric technic of HYMANS VAN DEN BERGH, they conclude that the measure-

¹ The studies presented here were aided by a grant from Frøken P. A. BRANDTS Legat.

ments of the yellow color are by far the most sensitive test for bilirubin — a conclusion which is certainly not justified.

HEILMEYER (1931) found the spectral maximum for pure bilirubin in chloroform at 450 m μ with $E_{1\text{cm}}^{1\%}$ 911—978 (average 949) and found that LAMBERT-BEER's law was valid for the wave lengths about the maximum when white light was used. The spectral region from 250 to 700 m μ was investigated. Solutions in alcohol showed a similar absorption curve but less steep and with the maximum at 450 m μ at which $E_{1\text{cm}}^{1\%}$ 843—877 (855). In aqueous solutions of alkali bilirubinates the spectral maximum was, however, found at 420 m μ with $E_{1\text{cm}}^{1\%}$ about 460; but this figure is not correct as the bilirubin is broken down so rapidly in these solutions that exact spectrographic readings are impossible with a technic like that used by HEILMEYER.

MÜLLER and ENGEL (1931, 1 and 2) found the maximum at 450 m μ with $E_{1\text{cm}}^{1\%}$ 918 for solutions in chloroform and the same maximum with $E_{1\text{cm}}^{1\%}$ 728 for solutions in alcohol. In aqueous solution they found the maximum at about 420 m μ but give no values for $E_{1\text{cm}}^{1\%}$ as it is impossible to carry out the determinations of the instable aqueous solutions with any degree of accuracy.

Pure aqueous solutions of bilirubin with addition of human serum were subjected to spectrographic measurements by MÜLLER and ENGEL (1931, 3) who found the maximum at 460 m μ against the 420 m μ of aqueous bilirubin solutions without the addition of serum. The $E_{1\text{cm}}^{1\%}$ at the maximum was 720. HEILMEYER and TOOP (1932) confirmed this observation and found $E_{1\text{cm}}^{1\%}$ 658 at 460 m μ . For horse serum they found the maximum at 470 m μ with $E_{1\text{cm}}^{1\%}$ 800. They further found that the absorption curve of the bilirubin in bile was identical with that of pure aqueous bilirubin solutions (*cf.* the findings of SHEARD *et al.*).

As seen from the above review of the literature, accurate readings of aqueous solutions are required, and previous measurements on aqueous solutions are only carried out at 400 m μ and longer wave lengths for which reason measurements at shorter wave lengths are required. As it is the instability of aqueous bilirubin solutions which renders accurate readings impossible, one has to use a technic requiring only a few minutes for the readings. Such rapid readings are possible in the visible regions with the Pulfrich photometer and similar instruments and in the ultra-violet and short-wave visible regions with the Hilger quartz spectrograph with Echelon cell outfit (*cf.* TWYMAN, 1933); the latter makes possible the exposure of a series of ten spectra within one minute of the preparation of the solution. In the following the technic and results of such readings are described.

Technic.

It is of great importance to carry out the measurements as soon as possible after the preparation of the solutions.

10 mg. (± 0.1 mg.) of bilirubin (preparation from the German factory "Homburg") was weighed off and dissolved in 1.5 ml. n/10 NaOH at room temperature and diluted to 10 ml. with buffer solution (pH 8) in a volumetric flask. This solution (100 m%) is stable for about an hour when stored in the ice box. Immediately before the reading 1 ml. of this solution was diluted to 100 ml. (*i. e.*, to a 1 m% solution) in a volumetric flask with buffer solution (most often pH 8). Readings were taken with the filters S. 43, S. 45, S. 47 and S. 50 of the Pulfrich photometer in $\frac{1}{2}$ cm. thick layer. For each filter four readings were taken after which the cuvettes were changed and four additional readings taken; the average of the eight readings constituted the final figure for the filter in question. From this figure the $E_{1\text{cm}}^{1\%}$ was calculated by multiplication by 2,000. During all the measurements the control cuvette was filled with water.

For the measurements of serum-containing bilirubin solutions 0.1—2.0 ml. (most often 1.0) of serum and 1.0 ml. of the concentrated bilirubin solution (100 m%) were mixed and diluted to 100 ml. with buffer solution (most often pH 7.3) in a volumetric flask. The readings and calculations were performed as for pure aqueous solutions. The sera used were all poor in bilirubin (below 0.5 m% determined with JENDRASSIK and GRÓF's method). In the case of three sera the dilution was performed with serum alone without water by dilution of 0.5 ml. of the bilirubin solution to 5 ml. with the serum in a volumetric flask and 0.5 ml. of this dilution to 5 ml. with the same serum in another volumetric flask. The bilirubin solution was in this way diluted 100 times with the use of only 9.5 ml. of serum. The readings were carried out as described above; in the case of the solutions of bilirubin in pure serum, however, with the exception that the serum used for the dilution was placed in the control cuvette.

The spectrographic measurements were carried out with a Hilger E 488 Quartz spectrograph with Echelon cell outfit.¹ For the readings of pure aqueous solutions a solution containing 5 mg. per 100 ml. was prepared by diluting the original concentrated solution 20 times in a volumetric flask. For measurements of serum-containing solutions dilutions containing 10 mg. bilirubin and 1 ml. serum per 100 ml. were used. In the control cuvette ("plain cell") a solution with exactly the same composition as the one in the "notched cell" but without bilirubin was placed (*i. e.*, the same percentage of serum, NaOH and buffer solution). In Fig. 1 a graphic illustration of the results is given; log. K is plotted against the wave lengths, and the curve thus is a

¹ The spectrograph of the Department of Pharmacy of "Dainmarks Pharmaceutiske Højskole" was used. For permission to use this instrument I bring Professor S. A. SCHOU, Ph. D., my sincerest thanks. I also wish to express my hearty thanks to S. IVERSEN, M. D. for his valuable assistance.

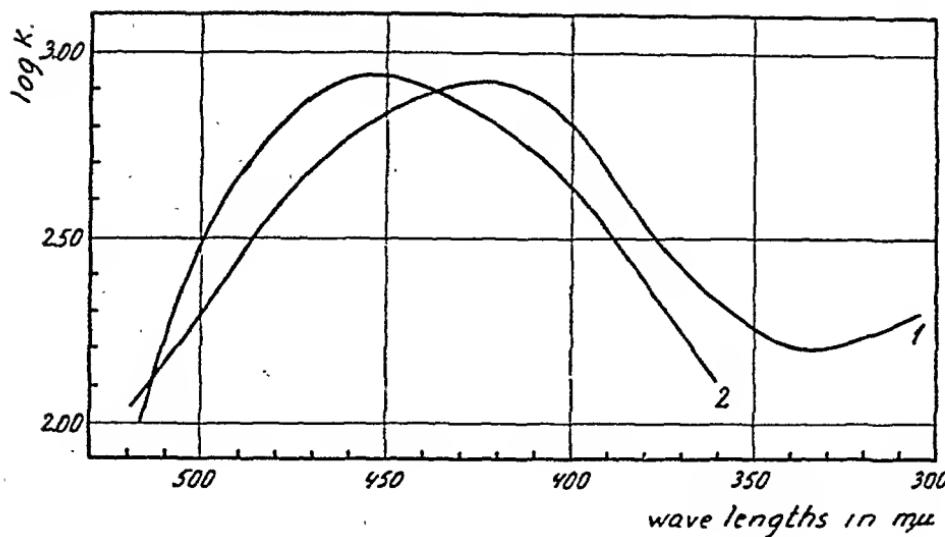


Fig. 1.

Spectral absorption of pure aqueous solutions of bilirubin (1) and aqueous solutions with serum (2).

so-called absolute color curve, the form of which is independent of the concentration and thickness of layer.

Table 1.

The $E_{1\text{-cm}}^{1\%}$ of Pure Aqueous Bilirubin Solutions with Different Filters of the Pulfrich Photometer.

Filter S. 43: 818; 782; 794; 808; 792; Average 799.

Filter S. 45: 756; 722; 730; 750; 748; Average 741.

Filter S. 47: 618; 634; 614; 640; 598; Average 621.

Filter S. 50: 228; 210; 218; 238; 230; Average 225.

E S. 45 divided by E S. 43: 0.92; 0.92; 0.92; 0.93; 0.94; Average 0.926.

E S. 50 divided by E S. 43: 0.28; 0.27; 0.27; 0.29; 0.29; Average 0.280.

Table 2.

The $E_{1\text{-cm}}^{1\%}$ of Serum-Containing Bilirubin Solutions with Different Filters of the Pulfrich Photometer.

Serum No.	1	2	3	4	5	6	7	8	9	10	Average
-----------	---	---	---	---	---	---	---	---	---	----	---------

Filter S. 43: 710; 722; 744; 748; 756; 738; 750; 728; 762; 718; 736.5

Filter S. 45: 810; 792; 806; 824; 830; 804; 812; 796; 840; 802; 811.5

Filter S. 47: 794; 788; 802; 808; 820; 810; 820; 786; 828; 806; 806.4

Filter S. 50: 420; 378; 354; 372; 406; 418; 420; 398; 432; 406; 400.3

E S. 45 divided by E S. 43: 1.18; 1.10; 1.08; 1.10; 1.10; 1.09; 1.08; 1.09; 1.10; 1.10; 1.10.

E S. 50 divided by E S. 43: 0.59; 0.52; 0.48; 0.50; 0.54; 0.55; 0.57; 0.54; 0.56; 0.56; 0.56.

Discussion.

As seen from Figure 1, our spectrographic measurements confirm the results of HEILMEYER and MÜLLER and ENGEL, but they also show that it is possible to obtain beautiful spectrographic curves of aqueous solutions, contrary to the belief of these authors. The maximum for aqueous solutions lies at $420 \text{ m}\mu$ with $E_{1\text{cm}}^{1\%}$ 825 and the corresponding values for the serum solutions are $455 \text{ m}\mu$ and 850. The curve of the serum solutions can roughly be constructed by displacing the curve for pure aqueous solutions $35 \text{ m}\mu$ towards the long-waved end of the spectrum. It is noticed that the $E_{1\text{cm}}^{1\%}$ at the maximum found by us for aqueous solutions is of the same magnitude as the values found by HEILMEYER and MÜLLER and ENGEL (*cf.* above) for chloroform and alcohol solutions. We have not performed readings of chloroform and alcohol solutions with the spectrophotograph as the measurements of the authors cited seem to us to give sufficient information in this respect. A single reading in the Pulfrich photometer showed the following values for $E_{1\text{cm}}^{1\%}$: S. 43 910; S. 45 1060; S. 47 920; S. 50 220 for chloroform and S. 43 816; S. 45 880; S. 47 750; S. 50 216 for alcohol. The alcoholic solution used was prepared from the chloroform solution and contained 5 per cent chloroform. These values are of the same order of magnitude as those reported by HEILMEYER and MÜLLER and ENGEL (measured in a König-Martens spectral photometer) but somewhat higher. As all sources of error except errors of weighing tend to cause too low values, our values are presumably the more correct.

The results of our readings in the Pulfrich photometer are presented in Tables 1 and 2. The values for $E_{1\text{cm}}^{1\%}$ found with the filter which gives the highest values are seen to be somewhat (3 to 5 per cent) lower than the values at the spectral maximum found by spectrography — a very natural finding. Similar to the findings with the spectrographic exposures the maximum absorption of the pure aqueous solutions was found at about $420 \text{ m}\mu$ (filter S. 43) whereas it was found at about $450 \text{ m}\mu$ (S. 45) for the solutions containing serum. The difference is unquestionable, and it is thus interesting to note that it can be detected by such a relatively simple instrument as the Pulfrich photometer. This difference can, by the way, be seen with the naked eye when a concentrated solution of bilirubin is mixed with equal amounts

of serum as its color becomes clear yellow while the pure solution — also when diluted with an equal amount of water — has a much more reddish appearance. This simple phenomenon seems not to have been described before.

The three sera for which the measurements were carried out, after dilution with serum without addition of water showed values for $E_{1\text{cm}}^{1\%}$ for all filters which deviated from the average values by a percentage below 5.

To find the cause of the different spectral curve in serum solution as against pure aqueous solution it was at first necessary to investigate whether the difference was due to variations in pH or was of other origin. Measurements of pure aqueous solutions gave the same $E_{1\text{cm}}^{1\%}$ between pH 7.5 and 13 for all four filters. With more acid reaction colloidal precipitation of the bilirubin took place. The serum-containing solutions showed the same $E_{1\text{cm}}^{1\%}$ for all four filters between pH 7.3 and 10, but at pH 13 (dilution with n/10 NaOH) the solution showed values of $E_{1\text{cm}}^{1\%}$ characteristic of pure aqueous solutions. In the interval pH 7.5—10 the characteristic difference between aqueous bilirubin solutions with and without serum thence is independent of pH and must be due to some substance or substances present in the serum. This was further confirmed by spectrographic exposures of pure aqueous bilirubin solutions and solutions containing serum made at the same pH (7.6). The same maxima as above were found (*cf.* Figure 1).

To find out the least amount of serum capable of causing the described alteration in the spectral absorption of aqueous bilirubin solutions, varying amounts of serum — 0.01—2.0 ml. — were added to 1 ml. of the concentrated bilirubin solution (containing 1 mg. bilirubin). Both the spectrographic exposures and the readings in the Pulfrich photometer showed the same extinction values and maxima for quantities of serum between 0.10 and 2.0 ml. added to 1 mg. bilirubin, but when smaller quantities of serum were used the absorption curve assumed a form more like that of pure aqueous bilirubin solutions. A serum containing 7.70 per cent protein and 3.94 per cent albumin showed on spectrography an absorption curve typical of bilirubin in serum solution if 0.1 ml. serum was used, but with 0.05 ml. serum the maximum was at 440 m μ and with 0.02 ml. at about 430 m μ . For another normal serum the quotient E S. 50 divided by E S. 43 was 0.60 when 0.05 ml. of serum was added to 1 mg. of bilirubin while it was 0.35

when 0.01 ml. was added (this quotient is about 0.28 for pure aqueous solutions; cf. Table 2). These observations show that when the amounts of serum added to 1 mg. bilirubin are below a certain limit (0.05—0.1 ml.) the absorption curve of the bilirubin-serum solution assumes a form which approaches that of pure bilirubin solutions the more the smaller amounts of serum are used. But even as low a quantity of serum as 0.02 ml. may give a distinct displacement of the spectral absorption curve.

As to the explanation of the absorption curve of the serum-containing bilirubin solutions it is natural to think of the combining of bilirubin with serum albumin demonstrated by several authors (SNAPPER and BENDIEN, 1931, 1938; BENNHOLD, 1932; PEDERSEN and WALDENSTRÖM, 1937; COOLIDGE, 1940) by means of various methods (diffusion experiments, ultrafiltration, cataphoresis and ultracentrifugation). It is to be looked upon as an established fact that bilirubin forms a firm and stable compound with serum albumin, that the reaction takes place rapidly, and that all bilirubin present in serum or added to serum is bound to serum albumin (PEDERSEN and WALDENSTRÖM; COOLIDGE). It seems to be a special property of serum albumin to form this compound as serum globulin and ovalbumin do not form similar compounds (PEDERSEN and WALDENSTRÖM).

It is now natural to assume that the formation of this bilirubin-serum albumin compound is also responsible for the alteration of the color and spectral absorption of pure aqueous bilirubin solutions after the addition of serum. Both the change of color and the formation of the compound in question is known to take place immediately after the addition of the serum, and the color is known to follow the albumin fraction of the serum proteins in experiments with diffusion, ultrafiltration, cataphoresis and ultracentrifugation. To prove that this assumption is correct it would be necessary to carry out experiments with pure, crystalline preparations of serum albumin; but as the author did not have such preparations at his disposal the experiments could not be carried out. As further serum albumin is not to be looked upon as a single well-defined compound, but represents a mixture of several substances, the problem is rather intricate. The "bilirubin-serum albumin" thence is not a well defined chemical compound, but the term designates that bilirubin forms firm and stable compounds with one or more of the individual compounds which together constitute the serum albumin.

Hitherto only human serum has been used in the experiments on the bilirubin-serum albumin compound as well as in the investigations into the spectral absorption with the only exception that HEILMEYER and TOOP (1932) found that horse serum caused a change in the spectral absorption of the bilirubin similar to that caused by human serum but with the spectral maximum at 470 m μ . The author therefore carried out investigations on serum from ox, horse, pig and dog with the technic described above and readings with the four mentioned filters of the Pulfrich photometer. Like human serum these sera all caused a distinct displacement of the absorption curve of the pure aqueous bilirubin solutions towards the long-waved end of the spectrum as seen from the quotients E S. 45 divided by E S. 43 and E S. 50 divided by E S. 43 which were of the same magnitude as for solutions with addition of human serum. The readings with the Pulfrich photometer did not allow the detection of minor differences in the absorption curves after the addition of the serum of different species, but the existence of such differences is proved by the above-mentioned spectrographic examinations of HEILMEYER and TOOP. As the changes in spectral absorption after the addition of serum must be considered due to the formation of bilirubin-serum albumin compounds, the formation of such compounds takes place in animals as well as man.

As 0.05—0.1 ml. of serum in one case was sufficient to transform 1 mg. bilirubin completely to the albumin compound while the transformation in another case was incomplete with these small amounts of serum (*cf.* above), it can be concluded that 0.05—0.1 ml. of serum lies near to the minimal amount of normal human serum which is able to transform 1 mg. of bilirubin. As the molecular weight of bilirubin is about 500 and that of human serum albumin about 65,000 while 0.1 ml. of serum contains ca. 5 mg. albumin it may be calculated that one molecule of albumin binds 25—50 molecules of bilirubin.

Summary.

Spectral absorption curves of aqueous bilirubin solutions with and without the addition of serum are given. Readings in the Pulfrich photometer showed good agreement with the spectrographic measurements. Solutions containing serum showed a maximum at 455 m μ , pure solutions at 420 m μ . The formation of a bilirubin-serum albumin compound is the cause of the change in the spectral absorption. Values for $E_{1\text{cm}}^{1\%}$ are given.

References.

BENNHOLD, H., Ergebni. inn. Med. u. Kinderheilk. 1932, 42, 273.
COOLIDGE, T. B., J. biol. Chem. 1940, 132, 119.
HEILMEYER, L., Medizinische Spektrophotometrie. G. Fischer, Jena, 1933.
HEILMEYER, L., Biochem. Z. 1931, 232, 229.
HEILMEYER, L. u. TOOR, P., Hoppe-Seyl. Z. 1932, 80, 603.
JENGRASSIK, L. & GROF, P., Bioch. Z. 1938, 297, 81.
MÜLLER, P. & ENGEL, L., Hoppe-Seyl. Z. 1931, 119, 117 (1); 200,
145 (2) & 202, 56 (3).
PEDERSEN, K. u. WALDENSTRÖM, J., Hoppe-Seyl., Z. 1937, 245, 152.
SHEARD, C., BALDES, E. J., MANN, F. C. and BOLLMANN, J. L., Amer.
J. Physiol. 1926, 76, 571.
SNAPPER, I. u. BENDIEN, W. M., Acta brev. neerland. 1943, 1, 4 and
Acta med. scand. 1938, 98, 77.
TWYMAN, F., Proc. Physical. Soc. (London) 1933, 45, 1.
YLPPÖ, A., Z. Kinderheilk. 1913, 9, 208.

(From the Medical Department A of the Rigshospital, Copenhagen.)

The Diazo Reaction of Bilirubin.

Measurements in Pure Aqueous Solutions and in Solutions containing Human Serum.

By

TORBEN K. WITH.¹

Received 9 July 1945.

Investigations on the diazo reaction of bilirubin have been carried out by several authors, but nevertheless certain questions concerning this reaction have not been sufficiently cleared up. For instance, is it not clear whether the reactions with different activators of the reaction (*e.g.*, caffeine and alcohol) are identical or whether minor differences in these reactions are found. Further, the spectral absorption has only been examined at certain values for pH, and the influence of serum addition — which causes considerable alterations of the spectral absorption of bilirubin (cf. WITH, 1945, 1) — on the diazo reaction has not been investigated.

The principles and the performance of the diazo reaction are not discussed here. A discussion may be found in earlier publications of the author (WITH, 1942 and 1944). In the following all extinctions are given as $E_1^1\%$. (the extinction of a solution containing 1 g per 100 ml of the reaction mixture — not of the original bilirubin solution — measured in a 1 cm thick layer). Alcohol and caffeine (*i.e.*, a saturated solution of caffeine, sodium bezoate and sodium acetate; cf. JENDRASSIK and CLEGHORN, 1937) were used as *activators for the reaction* and are in the following abbreviated to

¹ The studies presented here were aided by a grant from Frøken P. A. Brandt's Legat.

"alc." and "caff." respectively. The readings were performed at alkaline, neutral and acid *reaction* — abbreviated to "alk.", "neut." and "ac." respectively. The diazo reaction with caffeine as an activator read at acid reaction is thence written as "caff., ac." and the reaction with alcohol at alkaline reaction as "alc., alk."

Previous Investigations.

HEILMEYER and KRERS (1930) give a complete spectral absorption curve for "caff., ac." and "alc., ac." For the latter the value 1209 for $E_{1\text{cm.}}^1$ at maximum (570 m μ) can be calculated from the data. They also give readings with the Pulfrich photometer filter S. 57 from which the values 1330 for $E_{1\text{cm.}}^1$ (S. 57, "alc., ac.") is calculated as average of several measurements. For "caff. ac." the corresponding values were found to lie between 1010 and 1150. The extinctions thus were not quite identical for "caff., ac." and "alc., ac.", a finding which was further confirmed by the fact that the spectral absorption curves showed differences. But the authors did not carry out investigations to clear up whether these differences were due to differences in the pH or the composition of the solutions or really were due to the formation of different compounds by the two modifications of the diazo reaction.

BROCHNER-MORTENSEN (1934) found $E_{1\text{cm.}}^1$ 1224—1385 (average 1326) for "alc., ac." with S. 57 of the Pulfrich photometer. These values for $E_{1\text{cm.}}^1$ are calculated from his figures for the absorption constant (cf. WITH, 1945, 1). He adds caffeine, but first after the reaction has taken place, for which the reaction measured by him is to be regarded as a reaction activated by alcohol.

From the data of JENDRASSIK and GRÓF (1938, 1) the value 1460 for $E_{1\text{cm.}}^1$ for "alc., ac." with S. 57 can be calculated.

JENDRASSIK and GRÓF (1938, 2) give formulas for the calculation of the bilirubin concentration (in mg per 100 ml) from readings of the extinctions of "caff., alk.", "caff., neut." and "alc., ac." As the test solutions were diluted with the reagents respectively 5, 5 and 10 times in these reactions the values of $E_{1\text{cm.}}^1$ may be calculated from the formulas by setting the concentration to 1000 mg per 100 ml multiplied by the dilution figures mentioned. This gives the following values for $E_{1\text{cm.}}^1$: "caff., alk." with S. 61 941 (5000/5.32); "caff., neut." with S. 53 772 (5000/6.48); "alc. ac." with S. 57 1445 (10000/6.92).

It is seen that the values for $E_{1\text{cm.}}^1$ vary with the accelerator and the pH values used. The only value measured by more than one author is "alc., ac.", which shows some variation — and this is quite natural as the different authors do not use the same relative amount of alcohol, diazo reagent and hydrochloric acid. The extinctions of "alc., neut."

and "alc., alk." do not seem to have been measured by previous investigators.

Investigations on the diazo reaction of bilirubin solutions containing serum are only mentioned by JENDRASSIK and CLEGHORN (1937) and ROTHE (1939) who found that bilirubin added to serum was recovered quantitatively when the diazo-reaction was performed with caffeine as an accelerator and read in the Pulfrich photometer. Direct measurements from which the $E_{1\text{cm}}^{1\%}$ of the diazo reaction of solutions containing serum can be calculated are not found in the literature.

Remarks on the Technic.

The technic used for the readings in the Pulfrich photometer has been described in a previous communication (Witt, 1945, 1) in which also the importance of performing the readings as soon as possible after the preparation of the solutions was stressed. All readings were carried out in the so-called "Kleinkyvetten". A solution containing 5 mg bilirubin (from the German factory "Homburg") in 10 ml alkaline water was prepared and on this solution diluted 1:20 — i.e., a solution containing 5 mg per 100 ml — the diazo reactions were performed. The following reagents were used: "caff." 20 g. caffeine + 30 g sodium benzoate + 50 g sodium acetate diluted to 400 ml with distilled water; "dimix," (diazo mixture) a freshly prepared mixture of 0.25 ml (6 drops) of a 0.5% solution of sodium nitrite and 10 ml of a solution containing 5 g sulfanilic acid and 15 ml concentrated hydrochloric acid diluted to 1000 ml with distilled water; "Fehl." (Fehling's solution B) 10 g sodium hydroxyde and 35 g potassium-sodium tartrate dissolved in 100 ml distilled water; "HCl" pure concentrated hydrochloric acid; "alc." 96% alcohol; "H₂O" distilled water; "sol." designates the bilirubin solution used (5 mg per 100 ml).

The diazo reaction was carried out after the following reaction schemes:

"Caff., alk.": 0.1 ml sol. + 0.2 ml caff. + 0.05 ml dimix. + (after 10 min.) 0.15 ml Fehl.

"Alc., alk.": As caff., alk. with the exception that 0.2 ml caff. is replaced by 0.2 ml alc.

"Caff., neut.", "alc., neut." and "alc., ac." are performed in the same way as the corresponding alkaline reaction with the exception that the 0.15 ml of "Fehl." is replaced by 0.15 ml of "H₂O" for the neutral reactions and 0.15 ml "HCl" for the acid reactions.

"Caff., ac.": This reaction cannot be carried out with 0.2 ml caff. and 0.15 ml HCl as the caffeine reagent gives rise to precipitation with such a large amount of hydrochloric acid. This can be avoided by the use of the following scheme used by HEILMEYER & KREBS: 0.2 ml. sol. + 0.05 ml caff. + 0.1 ml dimix. + (after 10 min.) 0.6 ml H₂O and 0.05 ml HCl. The solution is diluted five times with this scheme as was the case with the others, but the amount of caffeine reagent used is only 0.05 ml.

to 0.2 ml of the solution against 0.2 ml to 0.1 ml of the solution in "caff., neut." and "caff., alk." — i.e., the caffeine concentration is only $\frac{1}{2}$ of that in the neutral and alkaline reaction with caffeine.

The reactions were performed in micro-test tubes ca. 40 mm in length and ca. 9 mm in inner diameter. The tubes were shaken after the addition of every reagent. After the addition of the dimix, an automatic clock was started which rang 10 minutes later, after which the last reagents were added. Within ten minutes after this the readings were performed.

As all readings were carried out with $\frac{1}{2}$ cm thick layer and the solutions all contained 5 mg per 100 ml and all were diluted 5 times with the reagents, the $E_{1\text{cm.}}^1 \%$ can be calculated by multiplication by 2000 of the extinctions read.

The solutions containing serum were prepared by mixing 1 ml serum and 1 ml concentrated bilirubin solution (100 mg per 100 ml) and diluting to 20 ml with buffer solution (pH 8) in a volumetric flask (cf. Wirth, 1945, 1). The solutions prepared in this way contained 5 mg of bilirubin per 100 ml. The same ten sera which were subjected to measurements in the author's investigations on the spectral absorption of bilirubin (Wirth, 1945, 1) were measured with regard to the diazo reaction, and the three sera for which the spectral absorption of bilirubin was determined after dilution with serum without addition of water were subjected to measurements of the diazo reaction after addition of bilirubin and dilution with serum alone. For these three sera the diazo reaction was measured both before and after the addition of bilirubin and the extinction of the reaction of the serum without the addition of bilirubin was subtracted from the extinction of the reaction of the serum after addition of bilirubin. By multiplication of the difference by 2000 the $E_{1\text{cm.}}^1 \%$ was found. Only "caff., alk." was measured for the solutions containing serum as alcohol and strong acids precipitate the proteins of the serum.

Table 1.

The $E_{1\text{cm.}}^1 \%$ of the Diazo Reaction with the Filters of the Pulfrich Photometer.

1. *Aqueous solutions of sodium bilirubinate (5 weighings).*

"Caff., alk."	with S. 61:	942;	946;	920;	984;	964;	Average	951
"Caff., alk."	with S. 59:	1070;	1148;	1088;	1124;	1080;	Average	1102
"Alc., alk."	with S. 61:	880;	900;	832;	844;	910;	Average	873
"Alc., alk."	with S. 59:	1162;	1148;	1106;	1180;	1154;	Average	1150
"Caff., neut."	with S. 53:	786;	824;	808;	774;	762;	Average	791
"Alc., neut."	with S. 53:	820;	826;	890;	918;	880;	Average	867
"Caff., ac."	with S. 57:	1126;	1076;	1058;	1040;	1032;	Average	1066
"Alc., ac."	with S. 57:	1384;	1370;	1448;	1352;	1376;	Average	1384

E (S. 61, caff., alk.) divided by E(S. 43,

spectral absorption): 1.15; 1.21; 1.17; 1.22; 1.22; Average 1.194

2. Solutions of bilirubin containing human serum (10 sera examined)

Serum No.	1	2	3	4	5	6	7	8	9	10	Average
"Caff., alk."											
with S. 61:	1026;	1053;	1104;	1089;	1050;	1086;	1020;	1084;	1064;	1072;	1065
"Caff., alk."											
with S. 59:	1140;	1167;	1173;	1155;					1170;		1161
E (S. 61, Caff., alk.) divided by E (S. 43, spectral absorption):											Average
	1.45;	1.46;	1.48;	1.46;	1.38;	1.47;	1.36;	1.50;	1.40;	1.49;	1.455

Discussion of the Results.

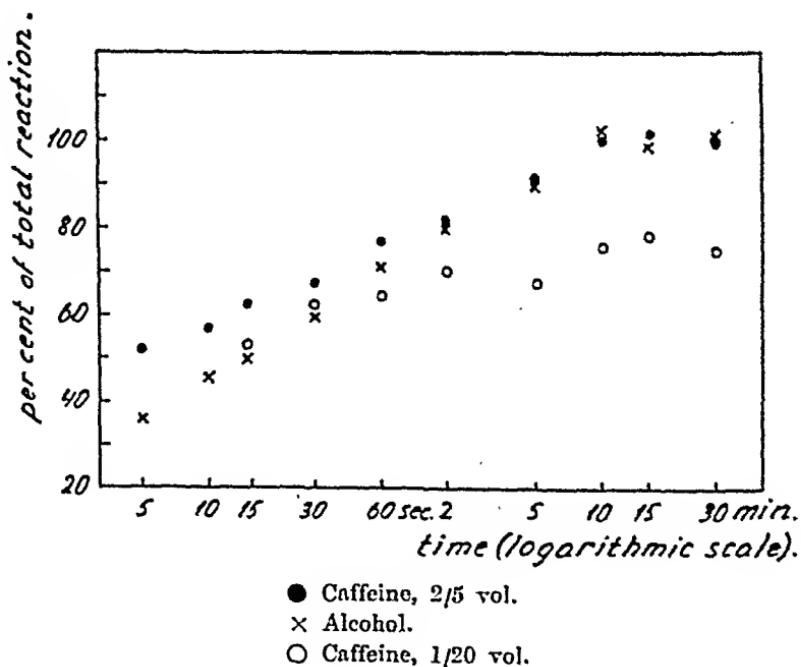
The values found for $E_{1\text{cm.}}^1 \%$ with the filters of the Pulfrich photometer which lay nearest to the spectral maximum of the various modifications of the diazo reaction are seen from Table 1. For the pure bilirubin solutions five different solutions were prepared and measured, for the serum solutions the figures for the mentioned ten sera are given. The spectral absorption of the bilirubin was measured for the same solutions and the results of these measurements are given by WITH (1945, 1). As the proportion between the diazo extinctions and the extinctions of the spectral absorption of the bilirubin (E S. 61 for caff., alk. divided by E S. 43 of the spectral absorption) is of interest in investigations on the origin of the yellow color of serum (cf. WITH, 1945, 2) the values of this proportion are also given in the table.

The values found for $E_{1\text{cm.}}^1 \%$ of the pure solutions agree well with those of previous investigators (cf. the values calculated above). The values with caffeine and alcohol as an activator for the reaction show distinct differences. The solutions containing serum — and presumably the compound bilirubin-serum albumin (cf. WITH, 1945, 1) — also show different values from pure aqueous solutions as $E_{1\text{cm.}}^1 \%$ (caff., alk.) is ca. 12 % higher for serum solutions than for pure ones with filter S. 61 and ca. 6 % higher with S. 59. Further, it is of interest that the proportion E S. 61/E S. 43 is 1.455 (average) for the serum solutions and only 1.194 for the pure aqueous solutions. This divergence between pure and serum containing solutions is also naturally explained as due to the formation of the compound bilirubin-serum albumin. It can be concluded that *in solutions containing serum not only the bilirubin itself, but also its azo-compound is bound to serum albumin*, and in this way the differences in the spectral absorption before and after

the addition of serum are explained in the same way for bilirubin and its azo-compound.

As $E_{1\text{cm.}}^1 \%$ for "caff., alk." is different for pure bilirubin solutions and solutions containing serum it is not correct to base the calculation formula for the serum bilirubin concentration on measurements on pure solutions of bilirubin as done by JENDRASSIK & GRÓF (1938). These authors give a formula which is most conveniently written $c = k (c_{1\text{cm.}} - k')$; $k = 5000$; $E_{1\text{cm.}}^1 \%$, c is reckoned in mg per 100 ml and k' is a correction for the diazo reaction of the caffeine reagent (commonly about 0.1; but is to be measured for each new reagent used. Cf. With, 1942; 1943). JENDRASSIK & GRÓF give the value 5.32 for k ; from our average $E_{1\text{cm.}}^1 \%$ for pure bilirubin the value 5.25 is calculated, but if the value for serum-containing solutions is used the value 4.70 for k is reached (all extinctions with the filter S. 61). So, the values calculated by means of the formula of JENDRASSIK & GRÓF must be about 13% higher than the real ones.

The three experiments in which all dilutions were carried out with the serum added to the concentrated bilirubin solution showed $E_{1\text{cm.}}^1 \%$ values within $\pm 5\%$ of the average value for the serum solutions measured after dilution with aqueous buffer solutions. This finding shows that bilirubin added to serum reacts quantitatively with the diazo reagent if caffeine is used as an activator and is recovered quantitatively in analyses based on the diazo reaction



in the modification of JENDRASSIK & GRÓF (*i.e.*, caff., alk.). That bilirubin added to serum is recovered quantitatively in measurements with the diazo reaction with caffeine was also found by JENDRASSIK & CLEGHORN (1937) and ROTHE (1939).

The Time Curve of the Diazo Reaction of Bilirubin.

If the $E_{1\text{cm}}^{\frac{1}{2}}$ of the diazo reaction with alcohol and caffeine respectively were not identical, one would be inclined to think that the development of the color showed differences with caffeine and alcohol. Examinations for this purpose were carried out by measuring the extinction of the diazo reaction 5, 10, 15 and 30 seconds as well as 1, 2, 5, 10 and 30 minutes after the reagent ("dimix.") was added. The measurements were carried out with "caff., alk." and "alc., alk." as well as with a modification of "caff., alk." containing only $\frac{1}{8}$ of the caffeine reagent of the original one, *i.e.*, with the same composition as "caff., ac." with the exception that "HCl" was substituted by "Fehl." (0.2 ml sol. + 0.05 ml caff. + 0.1 ml dimix + — after 10 min. — 0.5 ml H_2O + 0.15 ml Fehl.).

As the diazo reaction takes place only at a weakly acid reaction, the progress of the reaction is immediately interrupted by the addition of the alkaline "Fehl."-reagent. At first "caff." or "alc." and "sol." were placed in the micro-test tube and mixed and subsequently "dimix." was added very cautiously. In this way the "dimix" forms a layer above the mixture of the sol. and the activator, and the reaction does not set in before the two layers are mixed by shaking the tube. If the tube stands without shaking the reaction only takes place in the boundary zone between the two layers as a disk-reaction. If the time is taken with a stop-watch from the moment the tube is shaken, the beginning of the reaction may accordingly be regarded as fairly clearly defined. With the aid of an assistant the 0.15 ml "Fehl." was filled into the tube exactly at the end of the periods given above. As soon as the first drops of the strongly alkaline reagent had reached the solution in the test tube it was shaken, and the progress of the reaction thus interrupted practically simultaneously with the beginning of the addition of the "Fehl." In this way the time can be marked with the accuracy of about a second, and consequently the values after 5 sec. are rather inaccurate while the others are satisfactory.

Figure 1 shows that the diazo reaction both with caffeine and alcohol reaches its maximum after 10 minutes. The extinctions of the alkaline reactions measured at the different times are ex-

pressed in per cent of the values after 30 min. for alcohol and $\frac{1}{2}$ vol. caffeine, but for $\frac{1}{20}$ vol. caffeine they are expressed in per cent of the value for $\frac{1}{5}$ vol. caffeine after 30 min. It is also seen that the curves for caffeine and alcohol show considerable differences as the former reaches about 50 % of its final value within 5 seconds while the latter only reaches ca. 35 %. *The diazo reaction therefore develops more rapidly with caffeine as an activator than with alcohol* and the two modifications of the reaction cannot be fully identical.

Another point of some interest is the variation of the color of the reaction. All the alkaline and acid reactions with alcohol are of blue color while the alkaline reactions with caffeine are only blue after 5 and 10 sec., all the other alkaline caffeine reactions are green or greenish blue. This is due to the reaction of the caffeine reagent with the diazo reagent which is colorless when the reaction is neutral but clear green with strong alkaline reaction. As this diazo reaction develops more slowly than the diazo reaction of the bilirubin, the blue color of the latter is seen if it is interrupted after 5 and 10 seconds while the green color dominates if it is interrupted later.

The green diazo reaction of the caffeine mixture is only a negligible source of error in the reading of the diazo reaction of bilirubin in the Pulfrich photometer as its maximum spectral absorption lies at filter S. 43 and the absorption at S. 61 and S. 59 is very low. This was ascertained by performing the reaction with 1 ml $H_2O + 2$ ml "caff." + 0.5 ml "dimix." + — after 10 minutes — 1.5 ml "Fehl." and measuring in a 5 cm cuvette (the 5 ml of liquid is sufficient to fill a 5 cm "Kleinlyvette"); with the filters S. 50, S. 45 and S. 43 the readings were, however, performed in $\frac{1}{2}$ —1 cm layer. The extinction (calculated for thickness 1 cm) was only ca. 2 % of that of a bilirubin solution containing 5 mg per 100 ml with filter S. 61; with S. 53 it was ca. 5 % of the extinction of the diazo reaction of that bilirubin solution with S. 53, and with S. 47, S. 45 and S. 43 the extinction of the caffeine reaction was several times higher than the extinction of the bilirubin solution mentioned with the same filters.

The diazo reaction of the caffeine reagent may, even in spite of its low extinction with S. 61, cause errors in the determination of weak bilirubin solutions. In consequence of this the correction k' in the calculation formula given above has to be used. This correction is measured once for every caffeine reagent used by reading its diazo reaction in a 5 cm thick layer with S. 61 and dividing by 5 (cf. WITH, 1942, 1943).

The Influence of the Amount of Caffeine Reagent.

As mentioned above HEILMEYER and KREBS found a lower $E_{1\text{cm}}^1$ % for "caff., ac." than for "alc., ac." and their results are

confirmed by our readings (cf. Table 1). As pointed out, the amount of activator used in "caff., ac." is only $\frac{1}{8}$ of that used in the other reactions with caffeine, and it is therefore natural to assume that the low extinction of "caff., ac." is due to the small amount of activator used. This question was examined by measuring the alkaline caffeine reaction both with $\frac{2}{5}$ volume of caff. (the common reaction scheme for "caff., alk.") and with $\frac{1}{20}$ volume of caff. (cf. above in the section on the "time curve"). As seen from Figure 1 (cf. the 100 %-values after 10 minutes) the extinction with $\frac{1}{20}$ volume was only ca. 75 % of that with $\frac{2}{5}$ volume. As $E_{1\text{cm.}}^{1\%}$ at maximum (S. 57) for "caff., ac." (= 1066) was also about 75 % of $E_{1\text{cm.}}^{1\%}$ at maximum (S. 57) for "alc., ac." (= 1384) it is most natural to assume that the difference between these extinctions is caused by the insufficient amount of caffeine present in the reaction "caff., ac." (i.e., $\frac{1}{20}$ volume). The difference in $E_{1\text{cm.}}^{1\%}$ between "caff., ac." and "alc., ac." accordingly does not mean that the activating effect of caffeine on the diazo reaction is inferior to that of alcohol.

Figure 1 further shows that the diazo reaction with $\frac{1}{20}$ volume caffeine reagent reaches its maximum at the same time as that with $\frac{2}{5}$ volume. This does not agree with the view that caffeine acts as a catalyst, for if this were the case a reduction of the concentration with $\frac{1}{8}$ would cause a diminished velocity of the reaction but no alterations of the final result of the reaction — but the opposite takes place, i.e., unaltered velocity and a final extinction of only ca. 75 %. So, our results indicate that *the action of caffeine on the diazo reaction cannot be a true catalysis*, and consequently the term "activator" for caffeine and alcohol in relation to the diazo reaction has been used.

Are the Final Reaction Products of the Diazo Reaction with Caffeine and Alcohol Identical?

We have shown that the time curves for the diazo reaction with caffeine and alcohol show clear differences. But this does not necessarily mean that the final reaction products of the two modifications of the diazo reaction are different. The values for $E_{1\text{cm.}}^{1\%}$ (cf. Table 1) seem, however, to indicate that a difference exists, but as the solutions in which these values were measured were not identical in composition, minor differences in the extinction

values are not sufficient to prove a difference between the compounds present in the final solutions. To examine this problem, further measurements of entirely identical solutions of the reactions with alcohol and caffeine were performed.

The following solutions were prepared: 0.1 ml sol. + 0.2 ml caff. + 0.05 ml dimix. + — after 10 min. — 0.15 ml Fehl. + 0.2 ml alc. (= caff., alk. + alc.) and 0.1 ml sol. + 0.2 ml alc. + 0.05 ml dimix. + — after 10 min. — 0.15 ml Fehl. + 0.2 ml caff. (= alc., alk. + caff.). These two mixtures represent the two types of reaction and have exactly the same composition with the only exception of such differences which may be due to different end products of the two forms of the diazo reaction.

The results of these measurements of "caff., alk.+alc." and "alc., alk.+caff." are seen in Table 2. Here the extinctions with alkaline reaction are given. The bilirubin solution was the same as in the other experiments (5 mg per 100 ml) and the readings were carried out in a $\frac{1}{2}$ cm thick layer. As the solutions read were prepared by adding 0.2 ml to the 0.5 ml-volume of the original reaction mixture, the $E_{\text{1 cm.}}^1 \%$ is calculated by multiplication by $2000 \times \frac{7}{5}$.

Table 2.

Extinctions of the Diazo Reaction with Alcohol and Caffeine in Identical Solutions

Filter	S. 61	S. 59	S. 57
Alc., alk.+caff, extinctions of four solutions (5 m% ; $\frac{1}{2}$ cm)	0.268 0.281 0.272 0.290	0.315 0.345 0.322 0.341	0.282 0.312 0.307 0.313
Average	0.278	0.328	0.304
$E_{\text{1 cm.}}^1 \%$	778	918	851
Caff., alk.+alc. extinctions of four solutions (5 m% ; $\frac{1}{2}$ cm)	0.319 0.351 0.335 0.338	0.376 0.418 0.423 0.392	0.348 0.377 0.371 0.346
Average	0.336	0.402	0.361
$E_{\text{1 cm.}}^1 \%$	940	1126	1010

As seen from Table 2, four different solutions of bilirubin were measured and showed a deviation from the average value up to ca. 5%. Both reactions showed a maximum with the filter S. 59, but the extinctions were definitely higher for the caffeine reaction than for the alcohol reaction. If the $E_{\text{1 cm.}}^1 \%$ values are compared to those in Table 1, it is seen that the values for the caffeine reac-

tion are practically unchanged after the addition of the 0.2 ml of alcohol while those of the alcohol reaction become definitely lower after the addition of 0.2 ml caffeine reagent.

It seems consequently correct to conclude that the reaction products as well as the time curve for the diazo reaction differ for the two activators used. A possibility which also has to be taken into consideration is, however, that the reaction products formed in the two cases are the same, but that they are formed in somewhat higher concentration with caffeine than with alcohol as an activator. As the proportion between the $E_{1\text{cm}}^{1\%}$ for the two reactions is nearly identical (1.21—1.24) for all three filters, this possibility is by no means excluded.

For the further investigation of this point, measurements with a larger series of filters of the Pulfrich photometer were made with identical solutions both at alkaline, neutral and acid reaction (*i.e.*, "alc. neut. + caff.", "alc., ac. + caff.", "caff. neut. + alc." and "caff., ac. + alc." besides the two alkaline reactions mentioned) and so-called "typical color curves" were constructed. As the measurements for each solution were carried out with 8 filters, it is easily understood that they were very time-consuming and tiring, and that the extinctions obtained showed rather wide variation in duplicate analyses. The curves constructed did not show characteristic and constant differences between the reactions activated by alcohol and those activated by caffeine, but owing to the variation of the results the identity of the reaction products of the two reactions cannot be proved in this way. To do this it is necessary to perform measurements with a spectrograph in the visible region or with a photoelectric photometer — instruments which were not at the disposal of the author.

Summary.

The values of $E_{1\text{cm}}^{1\%}$ at the spectral maximum (filters of the Pulfrich photometer) are given for the diazo reaction after caffeine and alcohol-activator at strongly acid, neutral and strongly alkaline reaction. The values for solutions containing serum and pure aqueous solutions differ as the former are about 10 per cent higher than the latter. The higher values after the addition of serum are ascribed to the formation of the compound bilirubin-serum albumin. The proportion between the extinctions of the diazo reaction at its spectral maximum and the spectral absorption at the maximum of the yellow color showed almost the same value for bilirubin-serum albumin solutions prepared from ten different sera.

The velocity of the diazo reaction is definitely higher for caffeine than for alcohol (Figure 1). By determining the time curve for the caffeine reaction with $\frac{2}{5}$ and $\frac{1}{20}$ volume caffeine reagent it was made clear that the action of caffeine cannot be an ordinary catalysis.

Measurements of the alcohol and caffeine reactions in identical solutions showed definitely higher extinctions for the caffeine reaction than for the alcohol reaction. This difference between the two reactions is probably due to the formation of different reaction products, but this could not be finally proved.

References.

BROCHNER-MORTENSEN, K., Ugeskr. f. Læger 1934, *96*, 482.
HEILMEYER, L., Medizinische Spektrophotometrie. G. Fischer, Jena, 1933.
HEILMEYER, L., und W. KREBS, Biochem. Z. 1930, *223*, 352.
JENDRASSIK, L., und R. A. CLEGHORN, Biochem. Z. 1937, *289*, 1.
JENDRASSIK, L., und P. GRÖF, Biochem. Z. 1938, *296*, 71 (1) and 1938, *297*, 81 (2).
ROTHE, G., Z. ges. exp. Med. 1939, *106*, 338.
WITH, T. K., Nord. Med. 1942, *13*, 721.
—, Hoppe-Seyl. Z. 1943, *278*, 120 (1) & Acta med. scand. 1943, *115* 542 (2).
—, Nord. Med. 1944, *21*, 601.
—, Acta physiol. scand. 1945, in press (1) and Acta med. scand. 1945, in press (2).



Obituary Notice.

ERIK WIDMARK.

1889—1945.

At his death on the 30th of April 1945 Professor ERIK WIDMARK had held the chair of medical chemistry at the University of Lund for a quarter of a century. Born on the 13th of June 1889 at Helsingborg, he entered the University of Lund in 1907 and finished his medical course in 1916. Two years later he obtained his M. D. and became lecturer ("Docent") in physiology, but already in 1920, when only 31 years old, he obtained the professorship in medical chemistry after the sudden death of IVAR BANG.

WIDMARK received his early scientific training in the physiological department at Lund under Professor THUNBERG and began to study cell respiration. Partly under the influence of OVERTON he also took up investigations on the chemical conditions necessary for the maintenance of the normal structure of cells and published several papers on these questions. Very soon, however, he displayed independence and originality. Thus during his summer visits to the zoological station at Kristineberg on the west coast of Sweden, he wrote an interesting paper on the water flow in the vascular system of the jelly-fish.

Already in 1914 WIDMARK began the work on indifferent narcotics which was to occupy much of his time for the rest of his life. He carefully worked out micromethods for the determination of acetone and ethanol, which have been extensively employed in different countries for both scientific and practical purposes. In his thesis for the degree of doctor of medicine (1917) he showed that free acetone is distributed fairly evenly in different organs and that it passes to the urine, as well as to the alveolar air, in accordance with the demands of simple diffusion. Later on (1919) he demonstrated that the fall of the acetone concentration in the blood follows a monomolecular reaction.

Corresponding results were obtained for ethanol; they were published in a series of papers and summarized in his valuable monograph "Die theoretischen Grundlagen und die praktische Verwendbarkeit der gerichtlich-medizinischen Alkoholbestimmung" (1932). Ethanol was found to be distributed over the whole body, its average concentration being about 0.7 of that in the blood for male and 0.55 for female subjects. The difference is explained by the greater percentage of fat in the female body. But whereas for acetone the rate of disappearance from the blood was in direct proportion to the concentration, with ethanol the fall was independent of the level. The same was found for methanol, but the rate of disappearance was much smaller. In a special study the ethanol concentration in blood and urine after death was followed. As a result of these various investigations, the medico-forensic use of ethanol blood determinations obtained a firm and reliable foundation, and WIDMARK himself contributed with studies on the correlation between clinical symptoms and the blood alcohol level.

This work of his was performed in the borderland between physiology and biochemistry, where WIDMARK felt especially at home. Numerous papers on vitamins and on nutrition also belonged to this domain. Thus WIDMARK observed that plants which had lost their capacity to produce chlorophyll and lipochrome were unable to synthesize vitamin A, and he further investigated school-children's requirements of fat-soluble vitamins. Other publications dealt with different kinds of bread, with the selection of food etc.

WIDMARK also published contributions in several other fields, e. g. on lactation hypoglycemia, on organic acids in urine, on oxalic acids in vegetables, on renal calculus, and he described a microburette and a new method for the quantitative extraction of fluids. He was, further, the author of numerous popular articles on current problems of the day. Much appreciated by his students as a teacher, he was able to stimulate many of them to scientific activity.

He leaves behind him the memory of an energetic scientist with broad interests and fruitful ideas.

G. Liljestrand.

A Geiger-Müller Counter Arrangement for Biological Research.¹

By

J. AMBROSEN,

The Technical University of Denmark, Physical Laboratory I.

B. MADSEN, J. OTTESEN and K. ZERAHN,

Institute of Theoretical Physics, University of Copenhagen.

Received 11 July 1945.

In later years, the indicator method developed by Professor HEVESY has initiated an extended use of artificially radioactive elements in biological research. This development claims a convenient apparatus for the measurement of radioactivity. Usually a Geiger-Müller tube counter connected with an amplifier and a recorder is employed. With the essential improvement of the tube counter, according to TROST (1937), new demands are raised to the registering arrangement.

Since the appearance of the work by HILDE LEVI (1941), a complete counting apparatus has been constructed in this institute, which has for a long time worked satisfactorily.

The present paper deals with the construction of the counter and the amplifier as well as the statistical problems involved in the registration; furthermore an arrangement for automatical changing of the samples is described.

I. The Geiger-Müller counter (by B. MADSEN).

1. For the recording of single α -particles RUTHERFORD and GEIGER (1909) have indicated an "electrical method" which proved to be of greatest significance as the first step in the develop-

¹ Dedicated to Professor NIELS BOHR on the occasion of his 60th birthday.

ment of the "counter", a very important measuring instrument in radioactive work.

Between a wire and a plate an electrical field with a great gradient is formed. The electrodes are enclosed in a gas of suitable pressure. When an ionizing particle passes through the field it is possible by a suitable choice of potential to obtain a secondary ionization due to collision, so that the voltage impulse is proportional to the amount of primary ions even though considerably amplified.

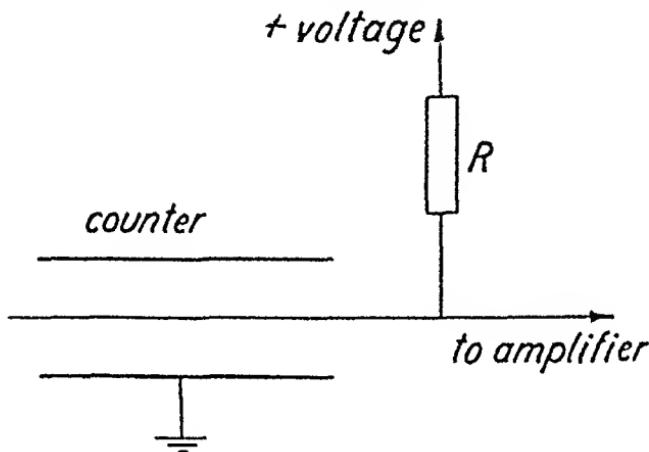


Fig. 1.

Counters based on this principle are applicable to measurements of radiation of intense ionizing, but not of β -particles.

In the peak-counter constructed by GEIGER (1913) which in its latest form, the GEIGER-MÜLLER tube counter (1928, 1929), has found extended use, the function is essentially changed. As before, the ionizing particle is sent into a field with a great gradient, now between a cylinder and a wire placed in its axis, but the voltage over the counter is chosen so as to exceed the limit of proportionality. As before, the ions are amplified due to collision, the factor of multiplication, however, being so great that the light of recombination forms photoelectrons in numbers sufficient to continue a stable discharge. If the voltage is reduced the discharge can be extinguished and, for this purpose, the resistance R is inserted (fig. 1). Used in this way the counter replies to any ionizing radiation, giving no information, however, regarding the magnitude of the primary ionization. The counter is working most satisfactorily when, as shown in fig. 1, the wire is made the anode. The connection between the range of proportionality and the range of real counting appears from the works by WERNER (1934).

If the filling gas is a pure gas (air or hydrogen), the space charge formed by the positive ions is of little influence and, to extinguish the discharge, the resistance R must have a value of about $10^9 \Omega$. This is inconvenient, for such high resistances do not keep their values constant, and further the time constant for the system counter capacity — series resistance is 10^{-2} — 10^{-3} sec. This quantity determines the resolving power of the counter.

2. Small admixtures of heavy vapours, especially alcohol vapour,

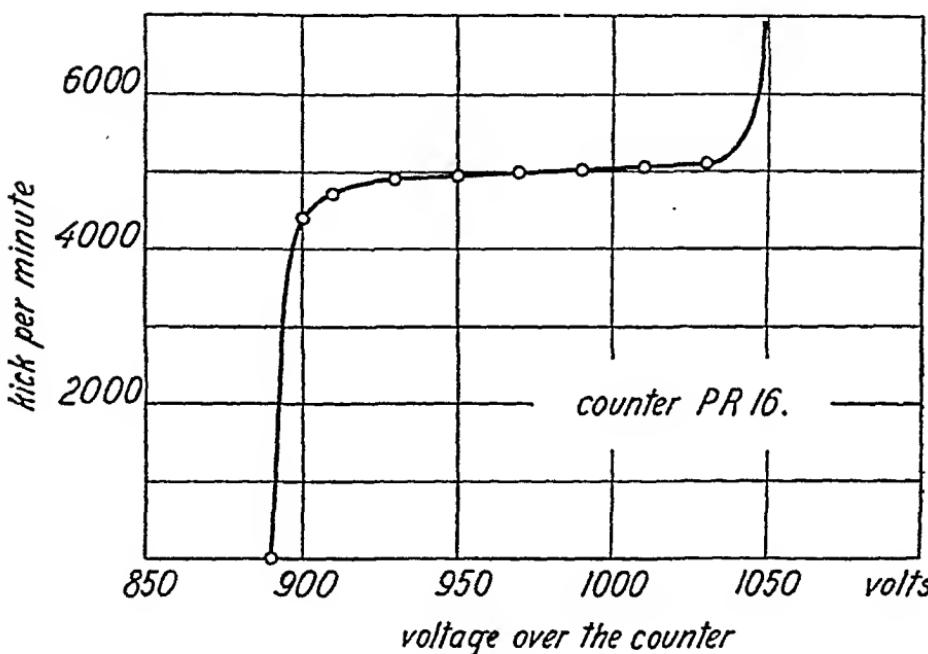


Fig. 2.

enable the ions to form a space charge sufficiently stable to extinguish the discharge. Then R can be made small, 1 — $10 M\Omega$. Now, the resolving time τ of the counter is a quantity associated only with the interior processes in the counter. The ordinary gas-tilling gives for counters of normal size $\tau \approx 10^{-4}$ sec. and still less for smaller counters. For the actual use of a counter, the knowledge of the counting rate as a function of the voltage is necessary. At a certain voltage the counter starts recording; with increasing voltage, the number of impulses increases rapidly to a value which is more or less constant over a certain range of voltage, until it finally increases again. In fig. 2 the number of impulses is shown as a function of the voltage for a counter of the type described in the following.

It is possible to define a "counting" (counting rate) only if the characteristic curve shows a constant range. In that case, the counting is proportional to the intensity of primary radiation, when losses due to the limited resolving power of the counter are taken into consideration.

If a particle passes through the counter in a boundary domain of weak electric field it is not recorded with certainty. The number of impulses within the counting range of voltage will then depend to some extent on the voltage, so that a completely constant range of the characteristic may not be expected, especially not for comparatively short counters.

After a counter discharge, there is a certain probability for the start of a secondary discharge, not due to ionizing radiation penetrating into the counter. This probability increases with the over-voltage (= counter v. minus start v.) and decreases rapidly with time. The secondary discharges afford the principal cause of the gradient of the characteristic curve.

The magnitude of the voltage impulses increases with the over-voltage, the increase being strongest at the beginning of the characteristic. The starting voltage of the counter (*i.e.* the voltage at which the counter begins recording) therefore depends in some degree on the sensitivity of the registering apparatus.

Details of construction.

3. TROST (1937) has investigated the behaviour of counters filled with gases of varying composition. The best counters were obtained with a mixture of 90 mm argon and 10 mm alcohol. Contamination by air within about 10% proved to have no essential effect.

Further, the cathode material — but not the anode material — is of importance for the quality of the counter. Brass is generally used, but slightly oxidized iron is just as suitable.¹ The secondary discharge effect can be somewhat diminished by gilding the inner side of the cathode.

With the use of counters for series measurements, often extended over a long time, claim is laid to stability and durability. This, *inter alia*, makes it necessary to use clean materials for isolation and cementing. Counters built with ebonite insulators and cemented

¹ The author is indebted to Mr. ZERAHN for kindly placing this result at his disposal.

with picein are not durable, because the materials give off gases and absorb the alcohol vapour, whereupon refilling soon becomes necessary.

When the radiation to be measured is a penetrating radiation, it is preferred to build the counter enclosed in a glass vessel which, after gas filling, is sealed off. If, however, the radiation consists of α -particles or slow β -particles, a thin window is indispensable. Mica is used on account of its considerable strength, and among

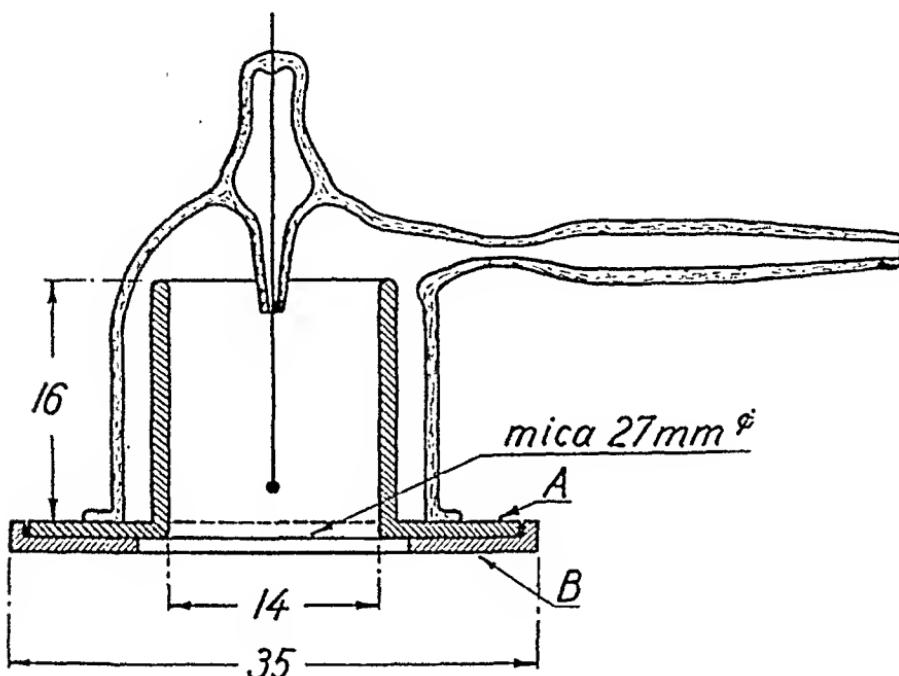


Fig. 3.

many cements selenium has been found to be most suitable. Other demands are: a large space angle and a small natural effect (zero count), so that a weak radiation can be measured. If no radioactive contamination is present, the natural effect is nearly proportional to the surface of the counter.

4. A handy construction is shown in fig. 3. On a tube of brass or compressed steel an edge is turned and over this a steel flange, A, is pressed. Between this and another steel flange, B, a mica window is cemented. Round the tube a cover of Jena glass (16^{III}) stands cemented upon A. The wire, a platinum wire 0.15 mm in diameter ending in a sphere about 0.6 mm in diameter, is introduced through and supported by a glass tube. A slight improvement in the correct measurement of the relative countings of samples of

greatly varying activities can be obtained by making the inner side of the mica window conducting; this can easily be done by burning gold on the mica. Then, the sphere on the counting wire must be placed 5—6 mm from the window. During evacuation and filling the side tube, narrowed for sealing off, is connected with the pump arrangement by a short thick-walled rubber tubing. Between the fillings with alcohol vapour and argon the connection is closed by a clip. The flange of the glass cover is ground to a plane by means of carborundum powder. After washing with water, the remaining grinding powder is removed by etching with fluorine acid. The mica window is 28 mm in diameter. With a free diameter of 14 mm windows weighing about 2 mg/cm² are used. Thinner windows will not safely stand atmospheric pressure.

5. Before the counter is assembled it is necessary to remove eventual radioactive contamination. Glass, mica and steel are wetted with alcohol and plunged into conc. nitric acid (the method is indicated by HOLM JENSEN (1944)). Brass is rinsed in dilute nitric acid.

The counter is assembled on a heated brass plate where a small crucible with selenium is also placed. It must be avoided that on the flange A so much selenium is smeared that it runs over the rounded edge. Hereby the window gets a sharp bending and rupture may occur. If any selenium vapour is condensed on the glass cover it may be removed by means of a small flame, while the counter is still hot.

After assembling, the counter must be cooled rather rapidly. By slow cooling the selenium solidifies in a grey crystallized form, and the joint glass-metal is probably not vacuum-tight. A suitable cooling rate is obtained when the counter is pushed from the hot plate to a cold brass block. The selenium then becomes smooth, black and viscous, and even after cooling the counter to 0° the joint remains tight. The flanges are made of iron on account of its small expansion coefficient.

Before the filling, the counter must be tested for tightness. Small leaks may be difficult to discover. In the finished counter they disclose themselves by a uniform rise in the start potential.

6. This counter has an effective space angle of $\frac{1}{3} \cdot 4\pi - \frac{1}{4} \cdot 4\pi$, the preparation being placed close to the flange B. If both the counter and its surroundings are free from radioactive contamination, the natural effect can be reduced to about 2 kicks/min. by shielding with 5—10 cm lead.

7. The counter voltage is usually taken from a high tension rectifying supply giving 2800 volts D.C.; 1260 volts is laid over a filter consisting of resistances and condensers. The remaining 1540 volts are stabilized by means of 11 small neon lamps. This high tension supply, which is much used in connection with G.-M. counters, gives a very constant voltage.

II. Amplifier and recorder circuit (by J. OTTESEN).

At normal working voltage the above described counter causes impulses of the magnitude of about 0.5 volt. As the scale circuit described later requires impulses of about 50 volts the amplifier must yield an amplification of more than 100 times. This amplification is most suitably obtained by the use of two stages. As mentioned before, the counter is self-extinguishing and can therefore be connected with a usual proportional amplifier, even if a small leakage-resistance is used. Nevertheless it has been found that in some cases it might be advantageous to make the resolving time of the amplifier, *i.e.* the time that passes from the moment the amplifier has reacted to an impulse till it can again react to an impulse, somewhat larger than the resolving time of the counter. In this manner some of the secondary discharges will be avoided in the countings. The probability that a discharge produced by an ionizing particle is followed by a secondary discharge increases strongly with the over-voltage and, in this way, a larger serviceable range of voltage and a smaller increase on the characteristic of the counter can be obtained.

An amplifier with a suitable resolving time is preferably obtained by means of a multivibrator circuit (JACOBSEN and SIGURGEIRSSON, 1943, GETTING 1938) and with a double pentode of the same type as the scale valves ELL 1 a very simple construction is obtained (fig. 4). The impulse of the counter is transferred to the control grid of the first valve. The voltage divider $R_1 - (R_2 + R_4)$ and the cathode resistance R_7 are adapted in such a way that the first valve amplifies reasonably; the anode current of the first valve is then about 3 mA. The grid bias for the second valve is chosen so that the anode current is nearly zero. A small negative impulse on the grid of the first valve will cause an increase in the anode potential of this valve. This increase is coupled to the grid of the second valve through the condenser C_1 with the result that the anode potential of the second valve decreases. This de-

crease in turn is transferred to the grid of the first valve mainly through the condenser C_2 , etc. The process continues until the anode current in the first valve is zero and the anode and the cathode in the second valve are at the same potential. By means of the voltage divider R_1-R_2 the control grid of the first valve is kept negative just as long as the anode voltage of the second valve is not altered. Therefore this state is steady until the potential of the control grid of the second valve decreases below the

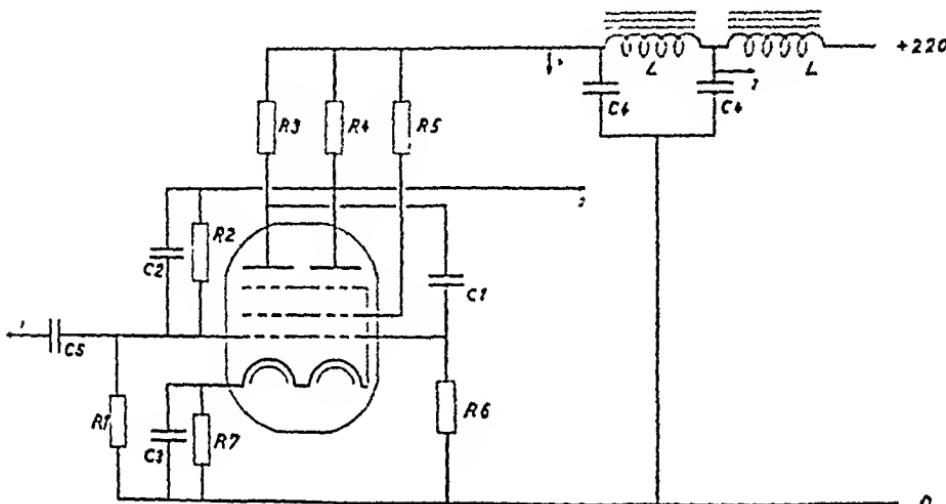


Fig. 4.

$$\begin{aligned} R_1 &= 0.05 \text{ M}\Omega \\ R_2 &= 1.2 \text{ M}\Omega \\ R_3 &= 0.03 \text{ M}\Omega \\ R_4 &= 0.05 \text{ M}\Omega \\ R_5 &= 0.5 \text{ M}\Omega \\ R_6 &= 0.1 \text{ M}\Omega \\ R_7 &= 0.002 \text{ M}\Omega \end{aligned}$$

$$\begin{aligned} C_1 &= 100-500 \text{ pF} \\ C_2 &= 3 \text{ pF} \\ C_3 &= 10000 \text{ pF} \\ C_4 &= 16 \mu\text{F} \\ L &= 4 \text{ Hy} \end{aligned}$$

Terminal 1 is connected to the counter wire.

Terminal 2 is connected to "in" by the first scale circuit.

Terminal 4 is positive voltage for the screen grid of the first scale circuit.

Terminal 3 delivers positive voltage for the rest of the scale circuits.

cathode voltage by the charging of the condenser C_1 . Then the anode potential of the second valve is increasing. By means of the condenser C_2 this increase is transferred to the grid of the first valve, etc. The currents in the valves resume very quickly the same values as before the impulse arrived from the counter. The decrease in voltage of the anode of the first valve will produce a considerable negative voltage of the control grid of the second valve through C_1 , so that for a short time after the process, a much larger impulse than usual is required to make the amplifier react.

When C_1 has been discharged through $R_6 + R_3$, the amplifier will have completely recovered.

The resolving time of the amplifier is almost proportional to the capacity of the condenser C_1 . The resolving time of counter + amplifier is measured by means of 2 radium sources and a "Scale of 128" to 7×10^{-5} sec. with $C_1 = 100 \text{ pF}$ and to 3.6×10^{-4} sec. with $C_1 = 500 \text{ pF}$, the working voltage of the counter being 100 volts above the starting voltage. The method is described in the following chapter. The resolving time decreases a little when the voltage of the counter increases and with $C_1 = 100 \text{ pF}$ and an over-voltage of 220 volts it has been measured to 5×10^{-5} sec.

The smallest resolving time which can be used is determined by the counter. Many counters give a poor plateau and may be useless, when the amplifier has the small resolving time, obtained with $C_1 = 100 \text{ pF}$, because many of the secondary discharges then are counted; but with $C_1 = 500 \text{ pF}$ most of the secondary discharges are not counted and consequently a good plateau is obtained. As mentioned in Chapter I, the described counter joined together by selenium perhaps allows faster counting, when the cathode is gold-plated.

The amplifier has been tested with 4 different valves, which all worked with an anode voltage supply varying from 180 volts to 235 volts. The starting voltage of the counter is up to 15 volts lower at 180 volts than at 235 volts. At 210 volts the difference between the starting voltages was no more than 15 volts. If the condenser C_2 is omitted the amplification will depend more upon the voltage and may fail at 180 volts with the resistance values used.

The anode supply is obtained from the 220 volts d. c. mains. The noise impulses are removed by means of two filters, each including a choke and an electrolytic condenser. The filament supply is obtained from the 220 volts a. c. mains by means of a transformer. Here, the noise impulses are greatly diminished by two condensers, 10,000 pF each between both primary terminal and earth. The rest of the noise impulses are eliminated from the cathode by the condenser C_5 . Shielded in this manner the amplifier only counts the noise impulses produced by switches close to the amplifier, i.e. within a distance smaller than about 1 m.

The mechanical recorder is a standard telephone message register supplied by the Telephone Company "Automatic", Copenhagen. It is driven by a gas-filled triode. The discharge is quenched

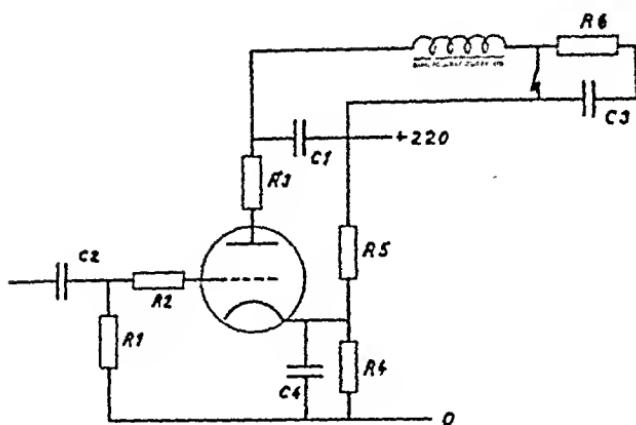


Fig. 5 a.

$R_1 = 0.5 \text{ M}\Omega$
 $R_2 = 0.5 \text{ M}\Omega$
 $R_3 = 2 \text{ k}\Omega$
 $R_4 = 5 \text{ k}\Omega$
 $R_5 = 25 \text{ k}\Omega$
 $R_6 = 200 \Omega$

$C_1 = 5000 \text{ pF}$
 $C_2 = 100 \text{ pF}$
 $C_3 = 0.1 \mu\text{F}$
 $C_4 = 2 \mu\text{F}$

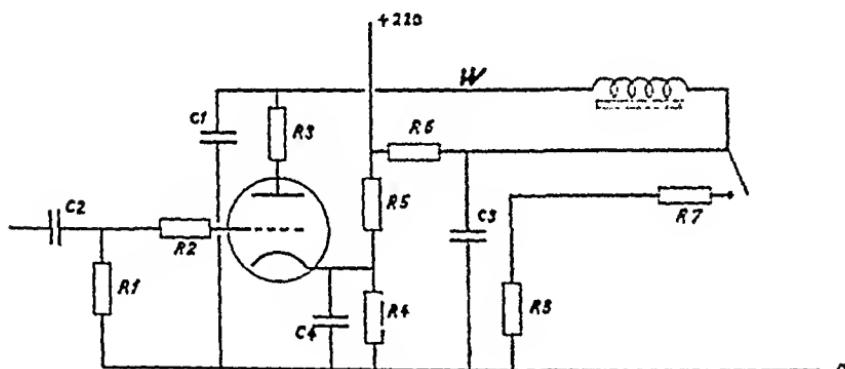


Fig. 5 b.

$R_1 = 0.5 \text{ M}\Omega$
 $R_2 = 0.1 \text{ M}\Omega$
 $R_3 = 500 \Omega$
 $R_4 = 5 \text{ k}\Omega$
 $R_5 = 25 \text{ k}\Omega$
 $R_6 = 5 \text{ k}\Omega$

$R_7 = 200 \Omega$
 $R_8 = 200 \Omega$
 $C_1 = 100 \text{ pF}$
 $C_2 = 100 \text{ pF}$
 $C_3 = 1 \mu\text{F}$
 $C_4 = 2 \mu\text{F}$

either by a "breaking" contact which interrupts the current or a "making" contact which decreases the anode potential to zero. In the circuits shown in fig. 5 a and 5 b the noise impulses produced by these processes are so much diminished that they do not interfere with the amplifier. In fig. 5 a this is mainly obtained by the condenser C₁ and the resistance R₅.

III. Scale circuit and statistic analysis (by J. AMBROSEN).

The resolving time for the mechanical recorder is much greater than for the counter. At higher rates of counting it therefore becomes necessary to diminish the number of impulses before they reach the recorder. This is accomplished by inserting between amplifier and recorder a number of stages of the "scale of two", introduced by WYNN-WILLIAMS (1931). Each successive stage divides the

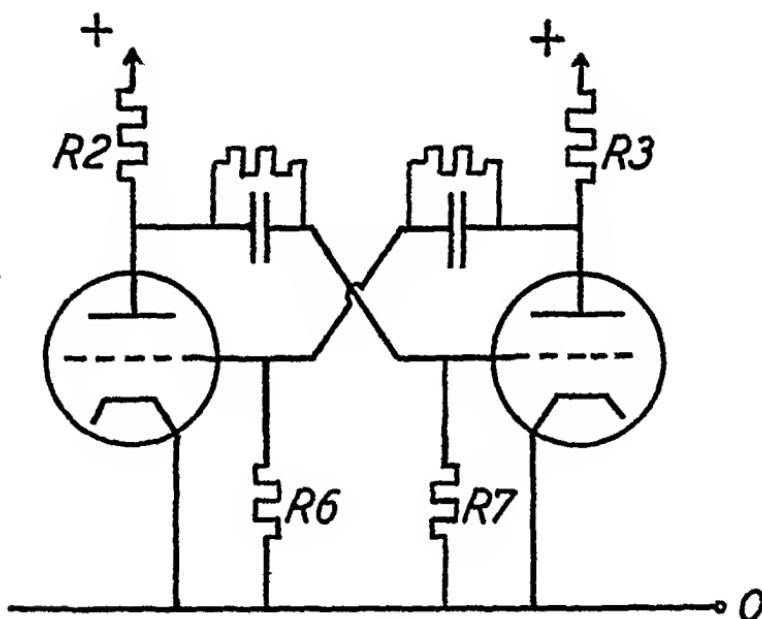


Fig. 6.

counting rate by two. The first "scales" employed Thyratrons: later diagrams utilize ordinary electronic tubes. (LIFSCHUTZ and LAWSON 1938.) Fig. 6 shows the principal features of such a system. It contains two tubes with the anode circuit resistances R₂ and R₃ and the grid-leak resistances R₆ and R₇. A resistance and a condenser in parallel are connected from each anode to the grid of the opposite tube. One tube will allow anode current to pass, so that its anode potential is low. Accordingly the grid potential is low in the other tube, which will not allow any anode current to pass. Using suitable values of the components the system has two stable states with anode current passing in one tube and the other tube non-conducting. The impulses to be counted are impressed on the grids in such a way that every impulse causes the system to jump from one of the stable states

206 J. AMBROSEN, B. MADSEN, J. OTTESEN AND K. ZERAHN.
 to the other. The potential on one anode will be raised by every second entering particle and lowered by every second; consequently the number of impulses transmitted to the next stage is only half the original number. In the diagram in question tubes are used of the type ELL 1, which contains two identical pentode-systems placed side by side in a normal-size bulb. This gives an easily wired and compact scale of two. (AMBROSEN, NIELSEN & SIGUR-

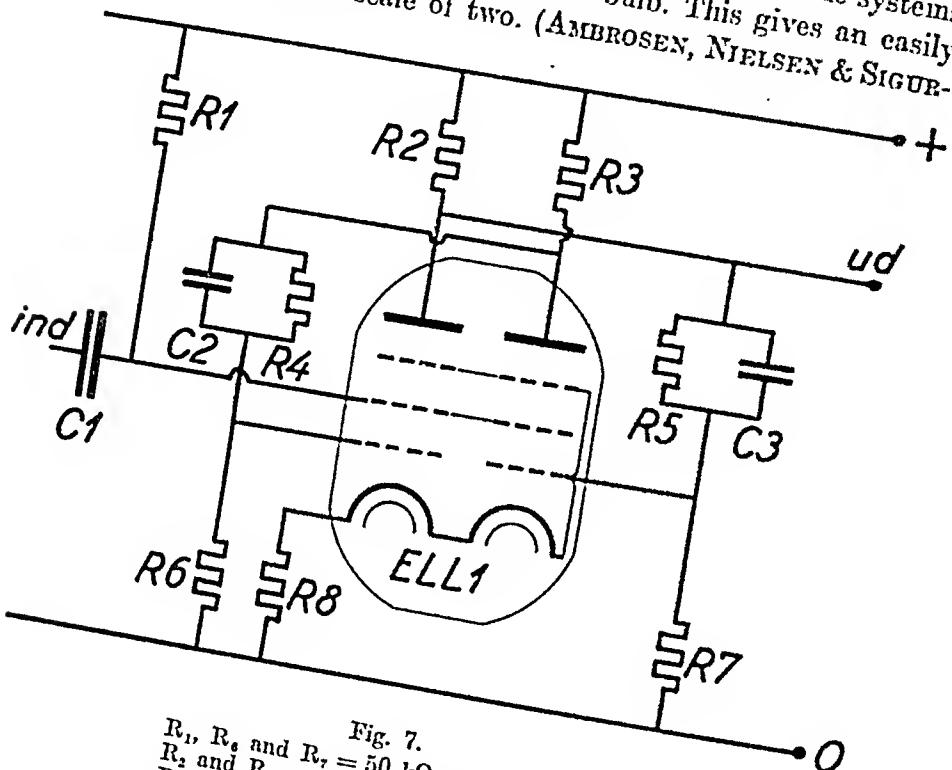


Fig. 7.
 R_1, R_6 and $R_7 = 50 \text{ k}\Omega$
 R_2 and $R_5 = 50 \text{ k}\Omega$ (2 Watt at least)
 R_4 and $R_8 = 100 \text{ k}\Omega$
 $R_3 = 3 \text{ k}\Omega$ (variable)
 $C_1 = 100 \text{ pF}$
 C_2 and $C_3 = 300 \text{ pF}$

GEIRSSON 1942.) The diagram with specifications below the figure is given in detail in fig. 7.

The entering impulses have to be negative and must have a size of about .50 volts. This rather great threshold value again assures that the circuit is not easily affected by electrical disturbances. It is possible to use a series of stages one after the other, all containing identical components.

Correct functioning is ensured by adjusting the cathode resistance R_8 : if this is too high, the system will be insensitive,

and if it is too low, the tubes will oscillate between the two stable states at a very high frequency.

This scale will work for a number of impulses pr. min. as high as at least 45,000. The time interval between two particles which can be separated by the system is therefore smaller than about $2 \cdot 10^{-5}$ sec.

Finally, we shall calculate the highest obtainable counting rate and the scaling-down ratio necessary.

Let us assume that the number of impulses is on the average f pr. sec. and put $t = 0$ at the arrival of one particle at random. The probability of getting at least one (possibly more) particle in the subsequent time t is:

$$P_1 = 1 - e^{-ft}. \quad (1)$$

For a counter, an amplifier or a register with the resolving time τ this formula gives the fraction lost in the apparatus, if τ is substituted for t .

In a similar way it can be shown that the probability of arrival of n (*i.e.* at least n , possibly more) particles in a time t is:

$$P_n = 1 - e^{-ft} \left[1 + \frac{(ft)^1}{1!} + \frac{(ft)^2}{2!} + \dots + \frac{(ft)^{(n-1)}}{(n-1)!} \right]. \quad (2)$$

For a register counting every n^{th} particle and having the recovery time τ , the formula (2) gives the fraction lost if τ is substituted for t .

P_n ($n = 1, 2, 4, 8, \dots, 128 \& 256$) is calculated for different values of ft and given in fig. 8 on a double logarithmic scale, and these will be used in the following calculations.

The apparatus in question has a tube counter and an amplifier interconnected in such a way that the joint recovery time is $\tau_1 = 4 \cdot 10^{-5}$ sec. The scale of two's and the register have $\tau_2 = 2 \cdot 10^{-5}$ sec. and $\tau_3 = 4 \cdot 10^{-2}$ sec., respectively.

We shall now allow a general counting loss in the apparatus of one per cent, a loss, which can easily be corrected for. Since τ_3 is much greater than τ_1 , which is again greater than τ_2 , we shall first deal with *the loss in the register*.¹

1. Curve 1 shows that if the register was connected directly to the amplifier a loss of 1 per cent was reached already when $ft\tau_3 = 0.01$ or $f = 1/4$ impulse per sec., which is a very slow counting rate. (It can be read from the curve too that an average of 150 impulses

¹ When a register is used at a high rate of counting the amount of the loss depends on the mechanical construction of the register (RUARK and BRANNER 1937). For losses which in accordance with our assumption are smaller than 1 pCt, however, the differences are negligible.

pr min. will be counted with a loss of 10 per cent, and perhaps it is not superfluous to stress, that what would be estimated as comparatively slow counting will show losses which by no means are negligible.

By inserting one single scale of two it is possible to increase the counting rate 15 times before reaching the one per cent-limit (curve 2). A second stage again improves the counting rate by a factor 5. After 3 stages $f\tau_s$ is equal to 3, which represents counting 4,500 particles a min. with 1 pct loss.¹

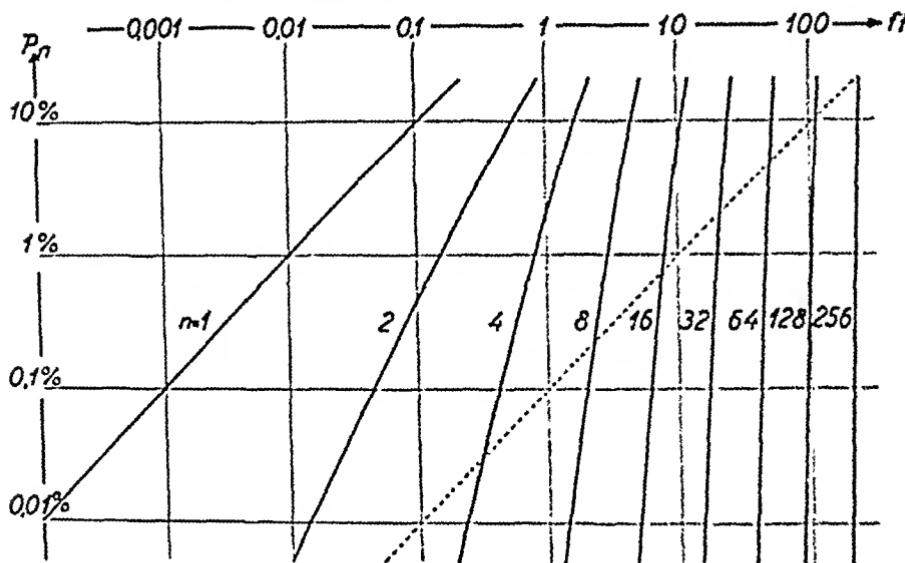


Fig. 8.

2. An increase in the number of "scale of two"-stages obviously improves the obtainable counting rate. But this cannot be extended indefinitely. At still greater counting rates the losses in the G. M. counter and the amplifier are no longer small, but reach the one percent limit.

The value of $f\tau$ for which thus occurs can be found in the following way: the ratio τ_s/τ_1 is equal to 1000/1; therefore the losses in

¹ The formulas were calculated under the assumption that the particles were statistically distributed in time; in fact the counter eliminates all intervals smaller than τ_1 . ALAOGLU and SMITH (1938) have discussed the alteration of the formulas necessary under this condition. τ_s being here much larger than τ_1 , the error however is negligible.

On the other hand, the counter cannot register n particles in a time smaller than $n\tau_1$ and for $n\tau_1 \geq \tau_s$, there are consequently no losses in the register. For a slow counter (airfilled without alcohol) is e. g. $\tau_1 = 5 \cdot 10^{-3}$ sec, also accordingly $\frac{\tau_s}{\tau_1} = 8$ and it is useless to have more than three stages of scale of two.

counter plus amplifier in the diagram are represented by the dotted line which is found by shifting the curve 1 with an amount of 3 units ($\log 1000$) to the right. $f\tau$ from the diagram is read to 10, which corresponds to 15,000 particles per min.

Counting a still higher number per min. means first that it is necessary to use 5 or more stages of scales of two but, nevertheless, a loss greater than 1 per cent is inevitable.

3. Finally the fact that τ is smaller than τ_1 shows that the scaling-down system under no conditions gives rise to losses in the counting.

It was shown in the foregoing that one scale of two will increase the speed of counting by a factor of 15 at the 1 percent-limit. That means that the fraction of particles lost is unaltered although the register counts now *7.5 times faster*. This surprising improvement is caused by the altered distribution in time of the impulses transmitted to the register. By increasing the number of stages the distribution becomes more and more regular, which means that it is possible to count with negligible losses a number pr. min. which nearly corresponds to the maximum speed of the register. A rather small increase in the counting will, however, then give considerable losses. This appears in the curves which for the higher values of n are nearly vertical.

By the method given above it is possible to find an approximate value of the counting loss for different numbers of particles pr. min. It is important, however, that the actual loss is checked by experiment. The usual way is the following: the number S_1 of particles pr. min. due to a radioactive source at a suitable distance from the counter is measured. Then the measurement is repeated for another source, with the result S_2 , and, finally, the number pr. min., S , is counted when both sources are used simultaneously. If S is smaller than $(S_1 + S_2)$ there is a loss, and the magnitude of the loss is also known. (The natural effect or "Zero-count" of the counter, without any radioactive source in the vicinity, of course must be subtracted in the above determinations.)

In this way the whole apparatus including counter and register can be controlled as a unity.

IV. Arrangement for measuring automatically (by K. ZERAHN).

1. In the study of biological problems by means of radioactive indicators a frequently appearing task is the comparative

measuring of radioactive samples. It is not a specially troublesome work, but it demands someone to change the samples and to read the mechanical counter. When many samples should be measured, it may be of importance to do this automatically. An arrangement answering this purpose is described in the following. The samples to be measured are placed in small aluminium dishes. The samples are changed when a clock makes a contact and each sample is measured on its own mechanical recorder. Fig. 9

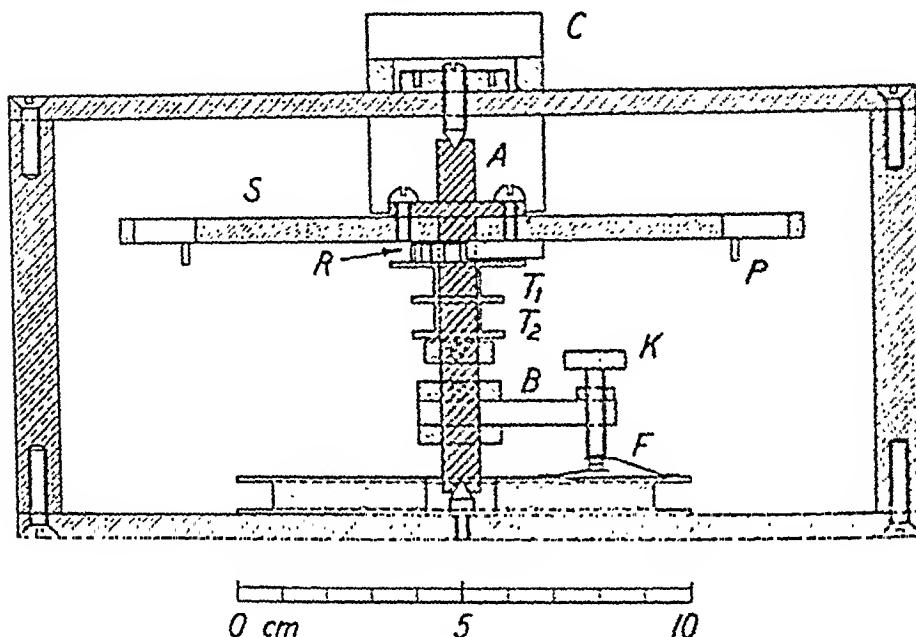


Fig. 9.

shows the apparatus in elevation, fig. 10 seen from above. The aluminium dishes are placed in 6 indentures in the circular disc S, and the indentures are placed in the corners of a regular hexagon. The disc S is firmly fixed to the shaft A which can move on two pin point centres.

Since the counting rate is largely dependent on the distance from the sample to the G. M. counter, the disc S has not to warp more than a fraction of 0.1 mm. The shaft A and the disc are moved by a weight of about 1 kg. suspended in a steel wire (not shown in the fig.). The steel wire is wound on a pulley T₁ which is fastened to another pulley T₂. On T₂ another steel wire is wound in the opposite direction of the first wire, holding a small weight. The pulleys are fastened to the ratched wheel R, and can so turn freely around the

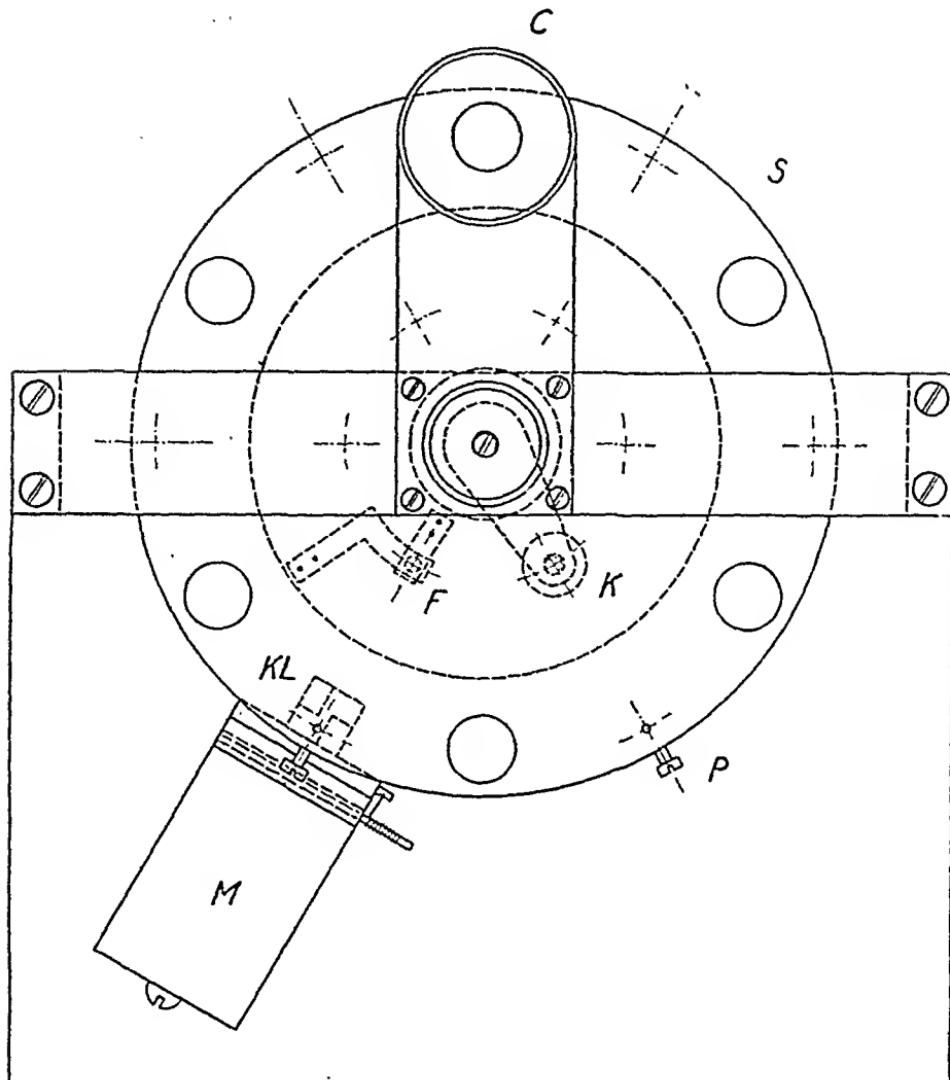


Fig. 10.

shaft in one direction. By pulling down the little weight the apparatus can be prepared for a fresh set of countings. In the border of the disc, 30° from each indenture, a steel pin is fastened by a small machine screw. The pin P will stop the movement of the disc by pushing against the stop KL. KL is mounted on the anchor of a small electromagnet M. One pin only can pass the stop when the anchor is attracted by the magnet. In an earlier paper this is described in detail (K. ZERAHN, 1945). The anchor is attracted when a synchronous clock connects two contact springs, e.g. every half hour. In order to avoid shaking of the samples one has to moderate

the movement of the disc; this may be done by connecting it with a piston moved in oil. Furthermore, an insulated radius B, fastened to the shaft, carries a screw K, which presses the contact springs F together (only one couple is shown in fig. 10). These contact springs connect the electrical circuit to the respective mechanical recorder. In fig. 5 b the conducting wire W is connected by the contact springs. The radius with the screw has to be placed on the shaft in

such a way that the screw connects a couple of contact springs when a corresponding indenture is right under the G. M. counter. This position must be taken up when a pin is stopped by KL. The G. M. counter is placed at C (fig. 10) and can be surrounded by a thick layer of lead. Mechanical recorders with "making contacts" are used because this is most convenient. The idea to use a mechanical recorder for each sample was suggested by Professor J. C. JACOBSEN.

2. When placing the radioactive samples in aluminium dishes and measuring them by pushing the dishes under a G. M. counter, only a fraction of the rays is utilized.

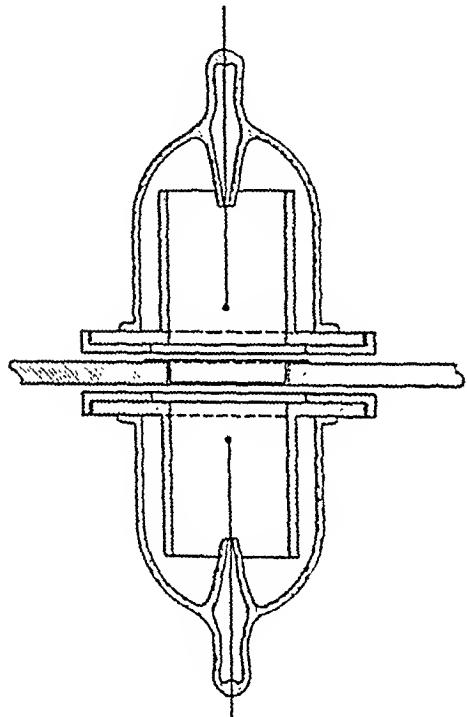


Fig. 11.

If the rays are not too weak, a greater part can be utilized by placing another counter under the aluminium dishes (*cf.* fig. 11). Each counter is connected with an amplifier of its own. Thus, the sample will be measured by the upper counter with an absorption of the rays caused by the thin mica window and the air. By the lower counter the absorption from 1/10 mm aluminium (thickness of the dish) will be added. If the rays from the sample are relatively hard the two counters will yield records of the same number, but if some of the rays are weak the upper counter will record relatively more. The use of two counters yields the following advantages: In the first place, two measurements of the sample are obtained simultaneously if the

rays are not especially weak. In the second place, the ratio between the results from the upper and the lower counter makes it possible to estimate the hardness of the rays from the sample and so perhaps to detect the presence of false activity. Finally, the statistical uncertainty will be diminished, in view of the increase in the total amount of recorded impulses.

Summary.

The present paper gives a detailed description of the construction of a GEIGER-MÜLLER counting tube, amplifier and recording device suitable for biological research. Furthermore, the statistical relations involved are discussed.

References.

ALAOGLU, L., and N. M. SMITH, Phys. Rev. 1938. *53*. 832.
 AMBROSEN, J., F. NIELSEN, and TH. SIGURGEIRSSON, Fysisk Tidsskr. 1942. *40*. 101.
 CURRAN, S. C., and V. PETRZILKA, Proc. Cambridge Phil. Soc. 1939. *35*. 309.
 GEIGER, H., Phys. Z. 1913. *14*. 1129.
 GEIGER, H., and W. MÜLLER, Ibidem. 1928. *29*. 839.
 GEIGER, H., and W. MÜLLER, Ibidem. 1929. *30*. 489.
 GETTING, I. A., Phys. Rev. 1938. *53*. 103.
 HOLM JENSEN, I., Lunds Univ. Årsskr. N. F. A 2. 1944. 40 Nr. 5. p. 8.
 JACOBSEN, J. C., and TH. SIGURGEIRSSON, Dansk. Vid. Selsk. mat.-fys. 1943. Medd. XX. Nr. 11.
 LEVI, H., Acta Physiol. Scand. 1941. *2*. 311.
 LIFSHUTZ, H., and J. L. LAWSON, Rev. Scient. Instrum. 1938. *9*. 83.
 RUARK, A. E., and E. E. BRAMMER, Phys. Rev. 1937. *52*. 322.
 RUTHERFORD, E., and H. GEIGER, Phys. Z. 1909. *10*. 1. — Proc. Roy. Soc. London. 1908. *A 81*. 141.
 TROST, A., Z. f. Phys. 1937. *105*. 399.
 WERNER, S., Ibidem. 1934. *90*. 384.
 WERNER, S., Ibidem. 1934. *92*. 705.
 WYNNE-WILLIAMS, C. E., Proc. Roy. Soc. London 1931. *A 132*. 295.
 ZERAHN, K., Acta Physiol. Scand. 1945. *9*. 346.

From the Institute of Theoretical Physics, University of Copenhagen,

The Measurement of Minute Samples with the Geiger-Müller Counter.¹

By

J. OTTESEN.

Received 11 July 1945.

When analysing organic compounds containing radio-phosphorus the sample is usually digested and one aliquot used for a colorimetric phosphorus determination, while the residual part is precipitated as magnesium ammonium phosphate and its activity measured with the Geiger-Müller counter. When determining the activity of small amounts (few mg or less) it may be of interest to determine the activity of the sample without digesting it. Such a procedure has the advantage that it makes it possible to carry out further purifications of the sample after the determination of its activity. In the usual measurements (LEVI 1941, AMBROSEN et al. 1945) aluminum dishes of 2.5 mm depth and 12 mm diameter are used in this laboratory. The ammonium magnesium phosphate samples which have a weight of about 100 mg entirely cover the bottom of the dish and a uniform distribution of such samples can easily be obtained. When trying to determine the activity of samples weighing few mg only, which do not cover the bottom of the dish, it was observed that the result obtained depended on the position which the sample occupies in the dish. At the suggestion of Prof. HEVESY the writer has worked out a method which overcomes this difficulty and simultaneously increases the sensitivity of the activity measurements.

¹ Dedicated to Professor Niels Bohr on the occasion of his 60th birthday.

Description of the Procedure.

We are using dishes having a diameter of 5 mm and a depth of 0.5 mm. The collar of the dish has a breadth of 3 mm. The dish is placed in a brass-ring denoted a in fig. 1, which fits in the sledge

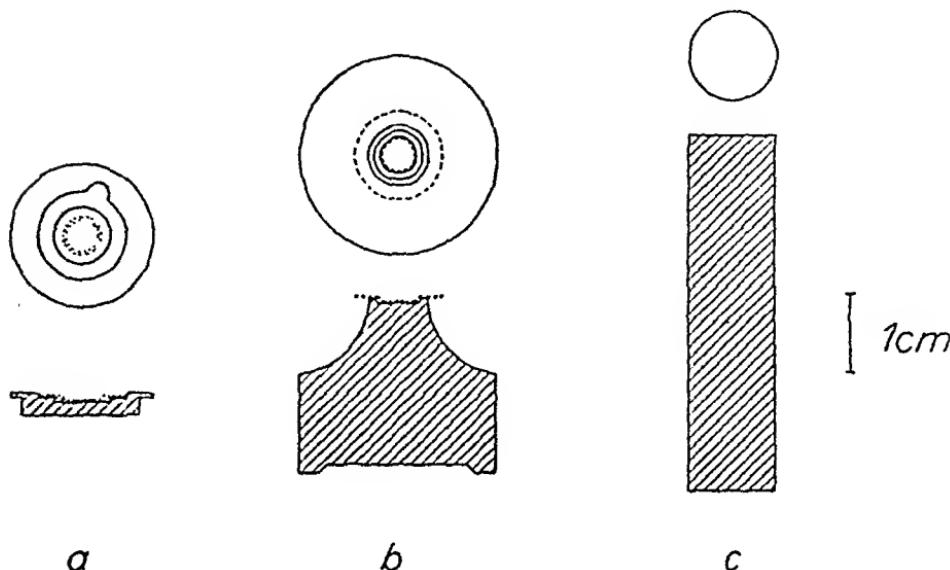


Fig. 1.

used in all our counting arrangements. To protect the sample an aluminum-foil having a diameter of 14 mm and a thickness of 0.01 mm is pressed on to the collar of the dish. The foil is pressed on to the dish in the following way: the dish is placed on a brass-holder shown by b in fig. 1 and after placing the sample into the dish the foil is pressed with two fingers against the collar of the dish. The fingers used to carry out this process are protected by rubber coatings. The final fixing of the foil is carried out by placing the dish in the brass-ring mentioned above and by pressing the foil with the cylinder denoted c in fig. 1. We obtain the weight of the sample, by weighing the dish after the aluminum-foil is fixed and by determining the weight of the empty dish and the foil.

We are interested to know the activity of the sample in percentage of the radio-phosphorus injected. To arrive at this figure a few mm^3 of the active solution is placed in an aluminum dish with the aid of a micro-pipette (HOLTER 1943) and obtain thus a

standard preparation. The dish is then treated as described above. While the weight of the standard preparation obtained by the last described treatment is negligible the weight of the nucleic acid sample may amount to some mg. In such a sample some absorption of the β -rays emitted by the active nucleic acid takes place. The error due to this absorption is determined in the following way: the activity of a standard sample of negligible weight is measured. The measurement is then repeated after placing 4 mg inactive nucleic acid above the standard sample. The activity is

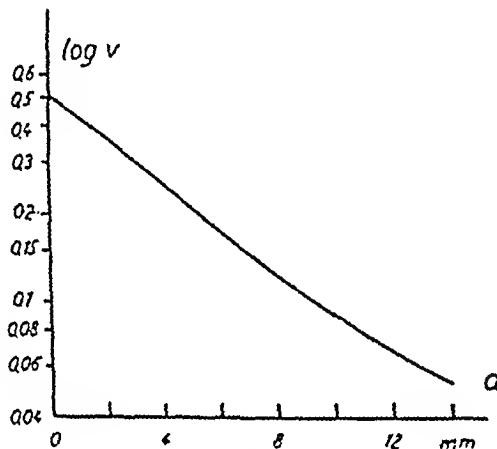


Fig. 2.

found to be reduced by 8 %. Assuming the absorption in a very thin layer of nucleic acid to be proportional to the thickness of the layer we find the average activity of the nucleic acid sample weighing 4 mg to be 4 % smaller than the activity of a sample of negligible weight.

It is of interest to note that this error is to a large extent compensated by the fact that by increasing the weight of the sample, the sample comes nearer to the window of the counter tube and produces a correspondingly higher effect on the counter. The extent of this effect can be estimated by the following consideration: we consider a point source at a distance a from the window of the counting tube, the diameter of the window being equal to d . As a measure of the β -radiation reaching the counter we consider the solid angle v . This is given by the equation:

$$v = \frac{2\pi \sqrt{a^2 + \frac{1}{4}d^2} (\sqrt{a^2 + \frac{1}{4}d^2} - a)}{4\pi (a^2 + \frac{1}{4}d^2)} = \frac{1}{2} \div \frac{a}{2\sqrt{a^2 + \frac{1}{4}d^2}}$$

When plotting the logarithm of v against a we get an approximately straight line as shown in fig. 2. It is indicated by the figure that by diminishing the distance from 7 to 5 mm the activity increases from 100 to 143, a result which was also verified by the experiment. The figure is furthermore indicating that a decrease of the distance of the preparation from the window by 0.25 mm leads to an increase in the activity registered by the counter amounting to 4 %.

While in the case of a sample weighing 4 mg the error due to the absorption of the β -rays in the sample is compensated by a corresponding increase in the amount of radiation reaching the counter due to the fact that the sample gets nearer to the window of the counting tube, this is no longer the case when the sample weighs for instance 1 mg. The correction to be applied for samples of different weight are stated below:

Amount of nucleic acid measured in mg	$\frac{1}{2}$	1	2	3	4
Percentage correction to be applied	- 3.5	- 3	- 2	- 1	0

The arrangement described is more sensitive than that formerly used in this laboratory and described previously. When measuring the activity of small samples e.g. 2 mg the number of counts registered by the counter arrangement described in this note is at least one and a half time as high as that registered by the former arrangement.

Summary.

A method is described which permits the quantitative determination of the radioactivity of minute samples.

References.

AMBROSEN, J., B. MADSEN, J. OTTESEN, K. ZERAHN. Acta Physiol. scand. 1945, in press.
 HOLTER, H., C. R. Lab. Carlsberg Sér. chim. 1934, 24, 399.
 LEVI, H., Acta Physiol. scand. 1941. 2. 311.

From the Laboratory of Zoophysiology, Copenhagen University.

The Effect of the Active Thyroid Substance on Metabolism and Other Functions in Guinea Pigs.

By

MARIE KROGH¹ and ANNA-LOUISE LINDBERG.

Received 7 July 1945.

In 1931 and 32 the present authors published papers in Danish concerning the physiological activity of iodine as combined in thyroxin and in normal and pathological thyroid glands. The activity was studied mainly by determinations of gaseous metabolism in young, but mature, male guinea pigs, weighing about 700 g. Apparatus and methods were described in some detail in the first Danish paper (1931) and summarily in English (1932). An extended series of experiments on different animals, including mice, pigeons, dwarf fowls and guinea pigs of different age, had shown the mature male guinea pig to be the most suitable, because he is easily accustomed to the experimental routine and then shows a remarkably constant metabolism averaging when the animals are in the postabsorptive state and perfectly quiet (standard conditions) 103 ml O₂/m² surface area/minute at a temperature, measured in the water bath surrounding the animal chamber, of 30°C. In the experiments then made the metabolism was determined before, during and after administering dried pig thyroids, human thyroids or different thyroxin preparations by mouth to the animals in a dosage of 7 × 4.5 mg iodine per sq.meter distributed over a

¹ In accordance with a wish expressed by MARIE KROGH before her death A. KROGH has taken upon himself the task of penning in English this report of a work to which his late wife has given much thought and labour. A small number of supplementary experiments were carried out in 1943 and 44 by the junior author.

fortnight. The surface area was calculated according to the formula $8.9 \sqrt[3]{\text{weight}}^2$. The constant 8.9 is taken from Tabulae Biol. Berlin 1926. This administration caused a gradual rise in metabolism which reached in about a week a relatively constant level.

The determinations showed a somewhat lower activity for thyroxin as compared with dried thyroid glands, either normal or showing colloid goitre, while the effect of glands from exophthalmic goitre was distinctly less per unit iodine, and it was concluded that the iodine in the glands is normally present in an active form either more potent or more easily absorbed from the intestine than thyroxin, while in the exophthalmic goitre part of the iodine content, which is regularly lower, is inactive. The result that normal thyroid glands have a practically constant metabolic effect per unit iodine is at variance with earlier determinations (MÖRCH 1929), but is corroborated by later studies (JORPES 1933) and definitely confirmed by the assays recorded below.

These studies were continued year by year in connection with a routine assay of thyroid preparations. A couple of papers were published in Danish in 1936 and 37. The results of these will be briefly recorded here and the accumulated material will be further utilized to establish the relation between the dose of active iodine and the metabolic effect under the standard conditions described above.

In 1935 a comparison was made between the effects on body weight, metabolism, pulse- and respiration frequency of male guinea pigs 1) of the partly purified thyroid product, elityran, claimed to possess special weight reducing properties and containing 630 mg iodine per cent, 2) normal dried glands with 220 mg% and 3) thyroxin with 65,000 mg%. Of the impure preparations a quantity corresponding to 4.5 mg J/m² was given by mouth on alternate days, but of thyroxin 6.5 mg J/m² subcutaneously.

As seen from table 1 there is very little difference in the effects on the metabolism, pulse- or respiration rate, and no difference could be observed regarding the symptoms of thirst, loss of hair and nervous excitability. There is an inexplicable difference in loss of weight which appears definitely lower for elityran.

Although the thyroxin dose was larger than the others $\frac{6.5}{4.5}$ the effect is if anything smaller and it was therefore now decided to make a few more comparative determinations in which the same dose

MARIE KROGH AND ANNA-LOUISE LINDBERG.

(0.5 mg iodine/m²/day) was given 1) in the form of dried thyroid (preparation 4111) by mouth 2) as thyroxin by mouth and 3) by injection of a thyroxin solution subcutaneously. The thyroxin (100 mg in all) was most kindly placed at our disposal by Dr. E. JACOBSEN, chief of the laboratories Medicinalco and was by Lovens kemiske Fabrik carefully mixed with lactose, so that a suitable dose could be weighed out and dissolved in Ringer.

Table I.

Preparation	Iodine %	Initial weight g	Aver. age daily metab. kg	Average loss of O ₂ min. % of initial	During experimental period			Maxi. mal pulse rate/ min.	Change in respi. ration
					standard error	Aver. age metabolism ml	Maxi. mal pulse rate/ min.		
Elityran	0.63	640	1.2	155	211	300	103—133		
(by mouth every sec. ond day in doses of 4.5 mg J/m ²)		565	1.0	150	222	288	88—132		
Gl. thyr. sicc.	0.22	820	1.3	153					
(by mouth every sec. ond day in doses of 4.5 mg J/m ²)		770	1.1	148					
Kendall Thyroxin	65.0	605	1.6	155	231	300	88—135		
(subcutaneously every second day in doses of 6.5 mg J/m ²)		565	2.0	156	224	300	88—138		
		615	2.1	156	213	240	80—132		
		788	1.9	150	218	240	89—102		

4 animals were given the preparation 4111 for about 17 days each and showed substantially the same effect as found in table 7 viz. 28—39, or on an average 34 ml O₂ increase in metabolism. 4 other animals were given the same dose as thyroxin by mouth and the increases found were 25, 23, 32 and 24 ml O₂/m²/min with an average of 26.

6 animals finally were given the same thyroxin dose subcutaneously and showed increases of 28, 29, 25, 29, 23 and 27 ml O₂/m²/min with an average of 27. The metabolic effect of iodine in the form of thyroxin is therefore certainly smaller (about 75 %) than that of the iodine found in normal thyroid glands, and since the activity

¹ Normal metabolism 103 ml O₂/m²/min.

² Normal pulse rate 140—150.

of thyroxin is found to be just the same whether given by mouth or subcutaneously it seems legitimate to conclude that the difference has nothing to do with any change taking place during digestion or absorption which latter appears to be complete. This result in so far agrees with the finding of DRESSLER and HÖLLING (1940) on young guinea pigs of 300—500 g that they found thyroxin iodine subcutaneously administered to be weaker than dried thyroid iodine by mouth in the relation $3.7/4.4 = 84\%$, but in two experiments in which the thyroxin was given by mouth they

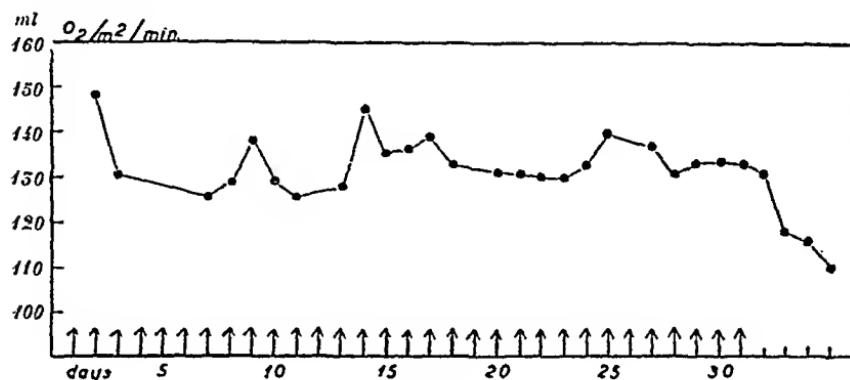


Fig. 1.

found a much lower activity ($3.7/10.4$). We have no explanation to offer for this striking discrepancy.

In 1936 the effect of α -dinitrophenol on metabolism, weight, pulse and respiration was compared with that of thyroid preparations and of thyroid stimulating extracts from the hypophysis. It was found that dinitrophenol in doses of 133 mg/m^2 given by mouth produces a rise in the metabolism which reaches a maximum of 160 — $180 \text{ ml O}_2/\text{m}^2/\text{min}$. in about an hour and falls again a few (about 4) hours later so that it is back to normal in less than 12 hours. A large dose (250 mg) causes a much higher rise (to 240 — 290 ml) which falls off more rapidly. The effect of thyroid or thyroxin sets in much more slowly and is maintained longer. By injection each day of the thyroid stimulating hormone the metabolism takes two days to reach a level of $140 \text{ ml O}_2/\text{m}^2/\text{min}$. and remains there until a couple of days after discontinuation of the injections (Fig. 1). The rise in metabolism is brought about mainly by an increased catabolism of carbohydrate, when this is available, as shown by determinations of the respiratory quotient, but when the glycogen stores are reduced fats are progressively

attacked. Dinitrophenol does not, as assumed by DUNLOP (1934), mainly stimulate the catabolism of fats. The protein metabolism is raised only slightly as shown by analyses of the urine. The combined metabolic effect of thyroid + α -dinitrophenol greatly exceeds the sum of the effects obtained by these substances separately, as shown by ALWALL (1936) for rabbits.

In 1937 the effects of inorganic iodine compounds and of di-iodotyrosine on simple hyperthyroidism and on experimental "Basedow" disease were studied in collaboration with H. OKKELS. The di-iodotyrosine was included, because it was claimed by ABELIN (1931) that this substance could reduce the effects of simple hyperthyroidism brought about by feeding thyroid to animals. Experiments were made on 4 groups of guinea pigs selected as before and having their normal standard metabolism determined.

- I. Hyperthyroidism was brought about by every day injections of a thyreo-stimulating hypophyseal extract and the "Basedow" thus produced treated with inorganic iodine.
- II. The same hyperthyroidism was treated with di-iodotyrosine.
- III. Hyperthyroidism procured by feeding thyroid was treated with inorganic iodine or
- IV. with di-iodotyrosine.

Body weight and metabolism of each animal was determined every day during the experimental period, and 24 hours after the last dose of hormone or thyroid the animals were killed and the histology and cytology of the thyroid examined by OKKELS, who was given no information regarding the experimental history and in reporting his findings referred only to the number given.

Group I comprises 11 animals of which 6 were given Lugols solution diluted to 1/10 and 5 a corresponding solution of potassium iodide. 5 mg J were given daily by mouth, but beginning a few days after starting the hormone injections as seen from the table. On one animal the injections and the iodine treatment were started simultaneously.

As shown in the table 2 the hormone injections alone cause a rise in metabolism of 20—30 %, but in spite of the continued injections the metabolism is reduced by the iodine and may even with potassium iodide reach values 10 % below the normal. The giving of iodine with every injection completely prevents the rise in metabolism.

The microscopic study of the thyroid shows that the accumula-

tion of colloid in the alveoli begins soon after the giving of iodine; while it takes a long time before the cells show a normal secretion picture. In one case the Golgi structures remained hypertrophied even after 44 days.

Table 2.

Weight of animal		Preparation	Number of treatments with		Average rise in metabolism		Morphology of thyroid ¹	
before treatment	after treatment		hormone alone	hormone + iodine	before iodine	two last days	Histology	Cytology
742	650	horm. + Lugol sol. 1/10	7	5	+35	+ 4	+(+)	++++ ¹
745	685	"	3	5	+36	+ 9	++	++++
670	605	"	4	8	+43	+14	++(+)	+++
730	740	"	5	44	+19	+ 2	0	++
725	648	"	0	8	- 2	0	(+)	
753	610	"	4	6	+16	+ 5	(+) el. 0	(+)
900	860	horm + KJ	9	9	+32	0	++	++
652	651	"	9	12	+30	- 7	0	0
629	545	"	7	18	+30	-10	0	+
681	690	"	9	33	+30	-10	-	+(+)
617	588	"	15	27	+22	- 3	-	+

Group II, table 3 comprises 4 animals in which the 5 mg iodine are given as di-iodotyrosine. The effect is the same in principle as that of inorganic iodine, but the metabolism takes longer to become normal and it would appear that the organic compound is less effective. It is interesting that two animals (one in each of the groups I and II) are only slightly affected by the iodine treatment in analogy with the well known clinical fact that certain cases of the genuine Basedow disease are refractory to the pre-operative iodine treatment.

When thyreo-stimulating preparations are regularly injected over several weeks the gland may in some animals become irresponsible. COLLIP (1934) takes this to be an indication of an anti-hormone formation. We have reason to believe that the mode of

¹ 0 means that the gland is morphologically normal.

The number of + signs indicates the degree of alteration in the direction of histologically: hypertrophy of cells, hyperæmia, absorption of colloid cytologically hypertrophy of Golgi structures with more highly developed ramification, basal and apical vacuoles, filiform mitochondria. The cells designated as +++ can scarcely be distinguished from those of a typical Basedow struma.

— means atrophic alterations of the thyroid gland and cytologically that the Golgi structures are reduced.

preparation and the purity of the hormone solution is essential in regard to the eventual production of refractoriness. The solution used in our experiments was prepared as EVANS' alkaline extract (1928) and we include a control experiment of 43 days duration. As shown in table 6 the gland remains sensitive to the hormone during the whole of this period. It is therefore legitimate to conclude that the fall in metabolism and the regressive alterations in the thyroid in our experimental animals are due to the iodine and not to any "antihormonal" effect caused by the hormone injections themselves.

Table 3.

Weight of animal	before treatment	after treatment	Preparation	Number of treatments with		Average rise in metabolism	Morphology of thyroid		Cyto-logy
				hor-mone alone	hor-mone + iodine		before iodine	two last days	
710	575		horm.+di-iodotyro-sine	13	21	+28	+21	+++	+++
675	580			13	21	+20	-4	0 el. (+)	0
815	710			9	16	+24	+4	0	+
815	810			9	16	+30	+3	0	+

Group III comprises 5 animals fed thyroid corresponding to 2.25 mg J/m²/24 hours for a fortnight and then in addition inorganic iodine for 6—7 days. As seen from table 5 the inorganic iodine has no reducing effect on the hyperthyreoidism produced in this way.

Table 4.

Weight of animal	before treatment	after treatment	Preparation	Number of treatments with		Average rise in metabolism	Morphology of thyroid		Cyto-logy
				hor-mone alone	hor-mone + iodine		before iodine	two last days	
630	505		Gl. Thyr. sicc.+Lugol sol. 1/10	14	6	+56	+70	0 el. (+)	+++
625	525			14	6	+56	+81	0	0
670	459			14	7	+41	+49	0	0
635	447			14	7	+43	+69	0	0
573	402			14	7	+50	+59	0	0

Group IV comprises 6 animals fed thyroid or elityran in the same doses as group III for a fortnight and then for some days in addition di-iodotyrosine corresponding to 30 mg J/m²/day. As seen from table 5 even this very large dose does not reduce the increased metabolism, and the thyroids of the animals presented a microscopic picture indicating a slightly reduced function as is the case with animals fed dried thyroid alone.

Table 5.

Weight of animal		Preparation	Number of treatments with		Average rise in metabolism		Morphology of thyroid	
before treatment	after treatment		hormone alone	hormone + iodine	before iodine	two last days	Histology	Cytology
835	590	Gl.Thyr. sicc. +di-iodotyr.	13	7	+64	+87	0	lost
535	375	,	13	5	+53	+70	—	—
570	435	,	13	5	+54	+72	0	—
565	480	Elityran + di-iodotyr.	13	4	+47	+35	0 or —	—
672	388	,	13	3	+29 ¹	+35	—	not exam.
758	600	,	13	8	+46	+51	—	,

Table 6 shows the results of a series of control experiments. The first of these was mentioned above. The two next give the well known results of feeding thyroid or elityran, and the rest show that iodine given alone will somewhat reduce the metabolism with slight and inconstant effects on the animals' weight and the histology of the thyroid gland. This metabolism reducing effect of iodine appears to be absent in the simple hyperthyroidism brought about by feeding thyroid, and this is probably due to the fact that the thyroid feeding, at the same time as it increases the "thyroxin" content of the blood and tissues, causes a diminished "thyroxin" output from the animals own thyroid. The metabolism therefore rises less than corresponding to the thyroxin administered. Judged by the histological picture viz. the flattening out of the cells and the accumulation of colloid (M. KROGH and H. OKKELS, 1937) the inhibiting action of thyroxin on the thyroid is similar in nature and extent to that of inorganic iodine, and this is probably the reason why neither inorganic nor organic iodine

¹ The small rise is due to emaciation from a diseased ventricle.

can exert any further metabolism reducing effect. In experimental "Basedow" conditions are totally different. The cells are high and show cytologically the signs of an increased secretory activity and the colloid is diluted and reduced or has disappeared completely. The effect of iodine can therefore make itself felt by causing accumulation of colloid, reduction of the height of the cells and a consequent decrease in metabolism, and this holds true whether the effect is direct upon the gland or --- as the work of FRANCK (1936) made probable --- indirect by way of the hypophysis.

Table 6.

Weight of animal		Preparation	Number of treatments	Average rise in metabolism two last days	Morphology of thyroid	
before treatment	after treatment				Histology	Cytology
755	640	hormone	92	+31	+++	+++
605	483	Gl. thyr. sicc.	17	+72	0	0 + -
640	548	Elityran	17	+57	0	-
665	673	Lugol sol. 1/10	16	+11	not examined	
652	643		10	+2		
688	710	KJ	17	+4	0	not examined
770	720		17	+10	0 or -	-
620	673	Di-iodotyrosin	14	+12	0	0
535	555	*	14	+19	0	0

In the attempt to evaluate the relation between dose and metabolic effect of dried thyroid glands the following procedure was adopted. Each series of metabolism determinations with a definite daily dose on one animal was plotted with the days as abscissa and the single feedings marked. Examples are given in figures 2, showing 2 "satisfactory" curves, one just acceptable and one discarded.

The determinations made before the thyroid treatment are utilized for an individual base line which never differs much from the general average. The thyroid feedings increase the metabolism which in most cases reaches a new, more or less constant, level in 2-6 days. Only such experiments are utilized in which it is possible to represent this level by a horizontal line drawn so as to average

* Usual dose \times 6.

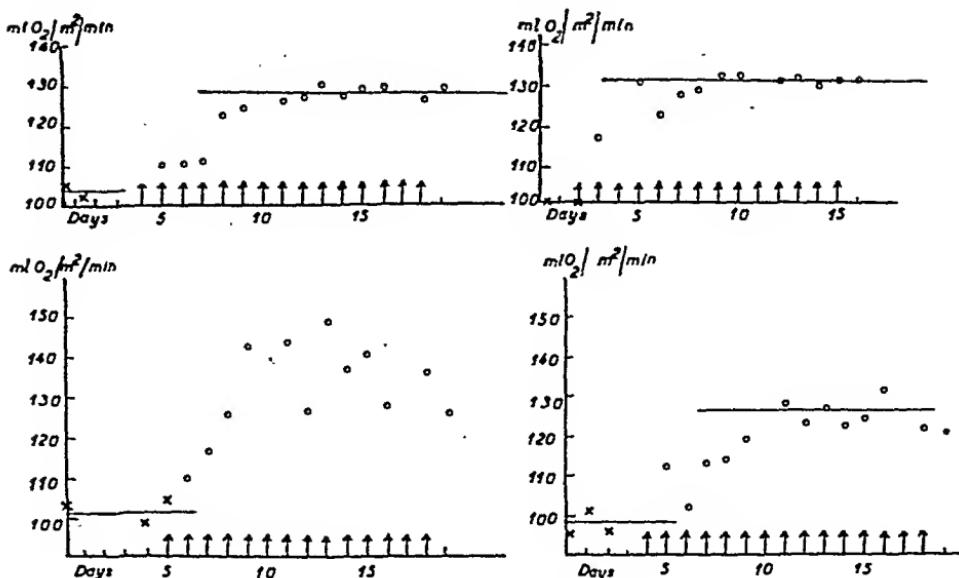


Fig. 2.

the majority of the determinations, but leaving out of account single aberrant results, especially such which are evidently too high. This is considered preferable to an algebraic average, because the thyroid treatment not only raises the true standard metabolism which we want to find, but tends to make the animals restless and to raise the metabolism by muscular movements.

A calculation was first made of the experiments concerning 4 different thyroid preparations, each of which had been tested repeatedly with widely differing dosage. The results are given in table 7.

It is evident that there is no systematic difference between the preparations; the "thyroxin" value must be very nearly the same for all. The average increases in metabolism for the doses 0.15, 0.25, 0.5 and 1.0 mg J/m²/day were calculated and as there is clearly no simple proportionality between dose and effect we tried the relation log dose/metabolic rise and this turned out as found also by GADDUM and HETHERINGTON (1931) and by DRESSLER and HÖLLING (1940) to be at least approximately a straight line for these doses as shown in fig. 3.

The whole material, comprising in all 4 determinations with the dose 0.15 mg J/m²/day, 17 with 0.25, 20 with 0.50 and 5 with 1.00, and representing 15 different preparations of dried pig thyroids with iodine contents varying from 0.15 to 0.26 % J was treated

Table 7.

Preparation No.	Dose mg J/m ²	Metabolism ml O ₂ /m ² /min.		Increase ml/m ² /min.
		before treatment	during treatment	
368	0.25	102	129	27
	0.5	100	136	36
	1.0	106	155	49
3710	0.25	93	120	32
	0.5	100	135	35
	0.5	107	142	35
	0.5	113	148	35
	0.5	102	134	32
	1.0	105	152	47
3610	1.0	109	160	51?
	0.25	106	128	22
	1.0	104	159	55
	1.0	108	154	46
4111	1.0	102	161	59
	0.15	95	112	17
	0.15	91	112	18
	0.25	102	128	26
	0.25	102	127	25
	0.5	103	139	36
	0.5	105?	149	44?
	1.65	100	155	55

in the same way, but 4 determinations with 0.25 and 5 with 0.50 had to be discarded, because no well defined metabolism level was obtained. The average standard metabolism before treatment was 100.1 ml/m²/min. \pm 3.4 and the mean error of the average of 42 determinations \pm 0.52, significantly lower than the formerly

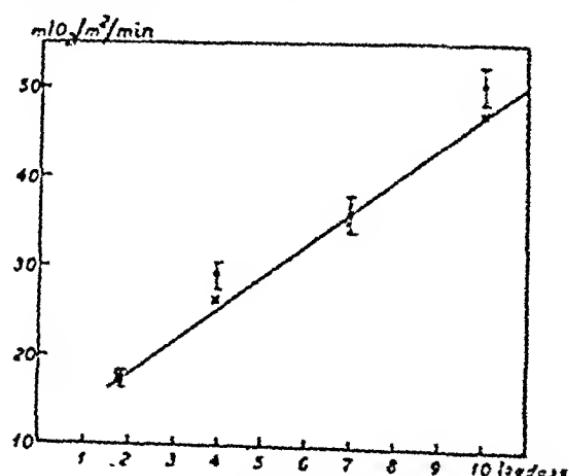


Fig. 3.

adopted average of 103. The average increases in metabolism and the mean errors of these averages were for 0.15 mg 17 ml \pm 0.8, for 0.25 mg 29 \pm 1.5, for 0.50 mg 35.7 \pm 2.1 and for 1 mg 50.4 \pm 1.8 when calculated from the normal metabolism as determined for each individual animal. These results are shown in fig. 3 with their mean errors indicated and are seen to be compatible with the straight line. When calculated as percentage increases from each individual normal the averages remain substantially the same, but their mean errors are definitely increased.

It is of course just as legitimate to calculate the individual increases in metabolism from the average normal of 100 ml O₂/m²/min. When this is done the average increases work out as follows: 0.15 mg 13 ml \pm 1.1, 0.25 mg 29 \pm 1.4, 0.5 mg 35.2 \pm 1.7 and 100 mg 51.2 \pm 1.0, figures which are still compatible with the line drawn, but fit it less well.

Summary.

Different preparations of dried thyroid from normal mammals and human beings and from cases of simple goitre have the same metabolic effect when given by mouth in equivalent daily doses based on the iodine content to mature male guinea pigs, accustomed to the metabolism determinations under standard conditions, while in exophthalmic goitre the iodine of the gland becomes less active.

A partly purified product, elityran, also shows the same effect, while pure thyroxin, even when given subcutaneously is definitely less active. No difference was observed between the activities of thyroxin when given by mouth or subcutaneously.

The metabolic effect of α -dinitrophenol given by mouth reaches a maximum in about an hour and falls again rapidly while that of thyroid preparations takes two days to develop and by administration each day reaches a more or less constant level, maintained throughout the period of administration.

Administration of iodine or di-iodotyrosine reduces the metabolism raised by every day injections of thyro-stimulating hormone (artificial "Basedow") and causes a regression of the histological and cytological "Basedow" symptoms in the thyroid gland, but has no effect on the hyperthyroidism brought about by feeding thyroid preparations.

Refractoriness to the thyreo-stimulating hormone was not observed in our case.

The quantitative relation between dose and metabolic effect of dried thyroid preparations was studied on 44 animals. On 9 of these no well defined metabolic level could be obtained. The 35 animals showed a fairly regular relationship. The average resting metabolism of the guinea pigs was 100.1 ± 3.4 ml $O_2/m^2/min.$. The increases were for 0.15 mg $J/m^2/day$ 17 ml $O_2/m^2/min.$, for $0.25-29.0$, $0.50-35.7$ and $10.00-50.4$. This corresponds to an approximately straight line relation between log dose and the absolute rise in metabolism.

Literature.

ABELIN, J., Klin. Wschr. 1931, 2, 2205.
ALWALL, NILS, Akademische Abh. Lund, 1936.
COLLIP, J. B., Ann. intern. Med. 1931, 8, 10.
DRESSLER, E., and K. HÖLLING, Arch. exp. Path. Pharmak., 1940, 196, 266.
DUNLOP, D. M., Brit. Med. J. 1934, 1, 524.
EVANS, H., and M. SIMPSON, J. Amer. med. Ass., 1928, 91, 1337.
FRANCK, G., Doktordisputats Kobenhavn 1936 (Danish).
GADDUM, J. H., and M. HETHERINGTON, Quart. J. Pharm. 1931, 4, 183.
JORPES, E. and Co-work, Nordiska Spec. Komm. Årsber. 1933, 30.
KROGH, MARIE, and ANNA-LOUISE LINDBERG, Bibl. f. L. 1931, 361
(Danish).
—, Acta path. microbiol. scand. 1932, 9, 21, II.
—, Ugeskr. Læg. 1935, 163 (Danish).
—, Hosp.tid. 1936, 387 (Danish).
—, and H. OKKELS, Acta path. microbiol. scand. 1932, 9, 37, III.
—, and H. OKKELS, Ugeskr. Læg., 1937, 863 (Danish).
MÖRCH, J. R., J. Physiol. 1929, 67, 221.

From B. A. Hjorth's department for metabolism research at the Wenner-Gren Institute.

Metabolism in Different Parts of the Brain, Especially in the Epiphysis, Measured with Radioactive Phosphorus.

By

ULF BORELL and ÅKE ÖRSTRÖM.

Received 8 June 1945.

According to investigations by HEVESY and HAHN (1940), a strikingly low metabolism is found in the brain, when measured by means of radioactive phosphorus; this we are in a position to confirm. Subsequent investigations furnished further evidence that there is but a low metabolism in the brain (HEVESY and OTTESEN, 1943). The authors isolated desoxyribonucleic acid from the brain and the metabolism proved to be very low even in this quantitatively considerable fraction.

This, however, does not exclude the possibility that a very high activity can be possessed by certain portions of the brain. The telencephalon, for example, will, by virtue of its great mass, obviously contain a greater percentage of P^{32} (radioactive phosphorus) — although it may exhibit a low metabolism — than small parts, e. g. the hypophysis or the epiphysis, even if these are very active. When the brain is examined as a whole, the possible high activity of these latter parts will therefore be hidden.

These results of HEVESY and co-workers are contradicted by experiments of HYDÉN (1943), who proved that the change of a nucleic acid fraction other than that investigated by HEVESY, namely ribonucleic acid, takes place very quickly in the brain, e.g. in the Purkinje cells of the cerebellum. HYDÉN measured the metabolism in the individual cell by CASPERSSON'S method (1936),

the wet weights of the different parts of the brain in different experiments agree very well with each other, which is a good criterion that the borders were similarly cut. The values given in this table represent the total weight of the parts concerned taken from three rats. An exception is made in one experiment, where only two epiphyses are used.

The phosphate and radioactive P contents of the respective parts were then determined. To burn the components and to liberate the phosphate from its organic combination, we proceeded as follows: 1 ml. of concentrated sulphuric acid was added to a brain component which had previously been rinsed down into a combustion flask of 50 ml. volume. The flask was warmed over a micro-flame until the material had been carbonized and dissolved. 2 ml. were used for larger pieces. When all had been carbonized, combustion was accelerated by the addition of a few drops of perhydrol. When the combustion was complete, a clear, colourless liquid remained. It was important to remove all traces of perhydrol by warming the neck of the flask. The solution was diluted with about 5 ml. of water and cooled. Some drops of phenolphthalein were added and the solution neutralized with 30% sodium hydroxide. The neutral solution was diluted to 25 ml., from which aliquot parts were taken for estimations of phosphate and radioactive phosphorus.

The total phosphate was determined by a modification of BRIGG's method (BRIGGS 1922, see also MÖLLERSTRÖM 1943).

The radioactivity of an aliquot was evaluated by a method elaborated by HEVESY and co-workers. The phosphorus is precipitated as a magnesium-ammonium salt and the precipitate is gathered in small aluminium dishes. The dishes are covered with a thin layer of shellac and dried in the oven at 110°. The impulses from the disintegrating phosphorus atoms are received in a Geiger-Müller tube and transmitted, via a system of amplifier valves, to a counting machine. For a more detailed account of the apparatus, reference is made to a work by LEVY (1941).

The values are expressed in relative specific activity. This implies that the number of impulses per unit total phosphate is first calculated.

$$\text{Specific activity} = \frac{\text{number of impulses}}{\mu\text{g total P}}.$$

Owing to the biological variations of the animals and to the fact that solutions of somewhat varying activities have been used in different experiments, we must refer the specific activity to some constant in order to obtain values comparable for different experiments. We have chosen the specific activity of the cerebellum as such a constant at 100 and the values for all other parts of the brain in the same experiment have been referred to it. Values thus obtained are called relative specific activities.

$$\text{Relative specific activity} = \frac{\text{specific activity of part of brain}}{\text{specific activity of cerebellum}} \times 100.$$

The cerebellum has been chosen because it can very easily be extirpated, as a consequence of which the wet weights are very constant from one case to another. Moreover, the cerebellum has a high proportion of

total phosphate and, absolutely speaking, considerable radioactive phosphorus, for which reason the analyses can be performed in an advantageous reading range. Changes in the activity of the cerebellum will, of course, be somewhat hidden by this procedure. On comparison of the specific activity of the cerebellum with that of other parts of the brain in different experiments, however, an idea can be gained of changes in the activity of the cerebellum.

Experimental Results.

The results of our experiments are compiled in Tables 2 and 3. Table 2 gives the phosphate contents of the brain components, expressed in per cent of wet weight. The table clearly shows that there are two groups of parts differing in their phosphate contents. The parts comparatively richest in phosphate are the epiphysis, the lobus anterior and posterior of the hypophysis, the plexus chorioideus and the substantia perforata, which contain between 1.31 and 0.59 per cent of phosphorus. The corresponding values for all other parts of the brain are between 0.42 and 0.31 per cent. Table 3 shows that the phosphate metabolism of the former parts takes place much more rapidly than that of the latter, except in the case of the substantia perforata, which exhibits very varying values. The values are given in relative specific activity (cerebellum = 100). In the table, the different parts of the brain have been grouped according to their activities into two very distinct groups. The first group contains very active parts of the brain. It consists of the epiphysis, the lobus anterior and posterior of the hypophysis and the plexus chorioideus. The other group comprises the twelve other parts tested. Finally, the relative specific activity of the blood also figures in the table.

The very powerful activity of the epiphysis seems to be the most remarkable phenomenon. The value of the relative specific activity exceeds that of the hypophysis in all cases. The mean value is about twice or three times the average value of the lobus anterior and posterior. We also gather from the table that no difference can be observed when the duration of the experiment changes.

As regards activity, the epiphysis is followed by the lobus anterior of the hypophysis. The lobus posterior also has a high activity, although this is generally somewhat lower than that of the lobus anterior. The results of the experiments with the lobi anterior and posterior are not surprising, considering the abundant production

Table 2.

Phosphorus contents of different brain parts.

Parts	Number of parts	Wet weight in mg.	P in µg.	P as per- centage of wet weight
Epiphysis	24	31.5	296	0.94
Lobus anterior hypophysis ..	33	197.0	1159	0.59
Lobus posterior hypophysis ..	33	45.7	599	1.31
Plexus chorioideus	33	58.6	542	0.02
Substantia perforata	30	57.3	367	0.64
Lobus olfactorius	30	1403	4624	0.33
Remainder of cerebrum	33	30562	95625	0.31
Thalamus	30	3244	12465	0.38
Anterior part of tuber cinereum	30	1125	3533	0.31
Posterior part of tuber cinereum	27	462	1757	0.38
Corpus mamillare	30	396	1586	0.40
Corpora quadrigemina	30	2061	7568	0.37
Cerebellum	33	7573	26210	0.35
Pons	30	3657	15420	0.42
Medulla oblongata	30	3626	14625	0.40

Table 3.

*Relative specific activities of different brain parts and blood in rats.
Specific activity of cerebellum = 100.*

Part	Interval between injection of P ³² and killing of animal						
	40 min.	90 min.	2 hours	5 hours	20 min.	45 min.	24 hours
Epiphysis	820	520	1710	775	2080	—	—
Lobus anterior hypophysis	258	410	600	565	652	390	640
Lobus posterior hypophysis	234	355	290	492	440	250	425
Plexus chorioideus	930	615	975	1040	420	485	255
Lobus olfactorius	90	124	180	125	200	136	—
Lobus occipitalis	70	72	150	86	98	92	—
Lobus parietalis	78	106	60	43	93	—	—
Thalamus	42	75	45	43	—	32	100
Anterior part of tuber cinereum	136	138	110	146	185	97	100
Posterior part of tuber cinereum	63	69	115	82	180	97	100
Corpus mamillare	50	—	95	111	140	80	142
Substantia perforata	63	—	85	68	233	59	325
Corpora quadrigemina	63	—	65	71	100	40	115
Cerebellum	100	100	100	100	100	100	100
Pons	63	56	45	50	—	54	100
Medulla oblongata	48	73	72	64	—	97	85
Blood	4350	2920	1910	2300	2550	880	410

Table 4.

Determination of turnover of acid-soluble and acid-insoluble phosphate fractions in epiphysis, lobus anterior of hypophysis and cerebellum from six rats.

Part	Precipitable with CCl_3COOH				Soluble in CCl_3COOH			
	P^m	γP	$P^m/\gamma\text{P}$	Relative spec. activity	P^{ss}	γP	$P^{ss}/\gamma\text{P}$	Relative spec. activity
Epiphysis	32	27	1.19	3970	217	14	15.50	2310
Lobus anterior hypophysis	20	57	0.35	1170	187	31	6.03	900
Cerebellum	113	3520	0.03	100	950	1415	0.67	100

of hormones in these parts. The synthesis of hormones is surely associated with a lively metabolism.

Nothing is known with certainty about the function of the epiphysis. It has been attempted to investigate the enigmatic function of the organ by extirpation, feeding, implantation and extraction experiments. Attempts have been made, moreover, to study possible histological changes in the epiphysis by the extirpation of other endocrine organs (cf. BARGMANN 1943). Certain conditions of pubertas praecox in man have been explained as a consequence of a reduction or cessation in the activity of the epiphysis (BERBLINGER 1932). To summarize the works on the function of the epiphysis, we may say that many authors have supposed a hormone activity in that organ, but have never proved this. The reason for these negative results probably lies in the use of unsuitable methods.

The question arises whether the phosphate metabolism in the epiphysis is connected with a lively turnover of nucleic acid or lipids or whether there is a vigorous metabolism of the phosphorylated intermediate links appearing in the carbohydrate cycle. In the former case, the metabolism might be based on a production of hormones directly associated with the protein or lipids; in the latter case, however, the hormone synthesis would come about by the aid of the energy available from phosphorylated intermediate links in the carbohydrate cycle. Table 4 shows that 90 % of the radioactivity is situated in the phosphate fraction soluble in trichloroacetic acid. Thus both the high activity of the hypophysis and that of the epiphysis depend on the fact that the acid-soluble phosphate is turned over much more rapidly than the phosphorus precipitable

by trichloroacetic acid. The vigorous metabolism of the active organs must therefore be regarded from the point of view that the energy necessary for the hormone synthesis is provided by a powerful carbohydrate degradation.

The function of the epiphysis is still as mysterious as before, but the present investigation clearly shows that this organ is a gland which must be placed on a level with the hypophysis. The methods we applied have for the first time furnished the means by which it may be possible to determine the function of the epiphysis. Investigations are being carried on in order to attain this goal.

The high activity of the plexus chorioideus is an interesting fact. Its activity often exceeds both that of the lobus anterior and that of the epiphysis. This, however, may depend on the fact that the organ is extremely rich in blood. The activity measured there may be composed of the activity of the organ itself and the injected radioactivity still unconsumed in the blood. Some experiments, however, indicate a lively metabolism in the plexus. Thus a very high oxygen consumption is found in the plexus chorioideus tissue (KREBS and ROSENHAGEN, 1931), whereas that of blood is very low. In consideration of the function that is ascribed to the plexus chorioideus, it is not amazing that the organ exhibits a vigorous metabolism. The epithelial cells of the plexus are said to produce liquor and to resorb certain substances from it.

One method of solving the problem is to analyse the velocity at which the different phosphate-ester fractions are turned over in the blood and in the plexus chorioideus. If the latter contains a preponderant quantity of blood, the rate of metabolism must be the same in the blood and in the plexus. Table 5 shows, however, that there is a considerable difference in this respect between blood and the plexus. The specific activity of free P in blood is

Table 5.

Specific activities of phosphate fractions, obtained after different times of hydrolysis, in the blood, plexus chorioideus and lobus anterior hypophysis.

Part	Free P	10 minutes hydrolysis	180 minutes hydrolysis	Residual fraction
Blood	11.95	2.33	0	4.47
Plexus chorioideus	3.85	1.13	1.00	0
Lobus anterior hypophysis	3.58	1.39	0.65	0

11.95 and is essentially higher than in the plexus chorioideus. This is quite natural, because the radioactivity has been injected intravenally in the form of free P^{32} . The fraction obtained after 180 minutes hydrolysis, chiefly hexose phosphate, exhibits a particularly low metabolism in blood, and, in the present case, no metabolism at all. These facts will be expanded by one of us in another paper. The metabolism in the corresponding fraction from the plexus, on the other hand, is high, the specific activity is 1.00. As regards the metabolism in the unhydrolysable residual fraction, the contrary is found, namely a high metabolism in blood and none in the plexus chorioideus. Thus the velocity of the turnover of various phosphate fractions is quite different in blood and the plexus chorioideus. Hence we may infer that it is not the blood in the plexus that causes its partially high activity, which must be due to the metabolism in the plexus tissue itself. Table 5 indicates, moreover, that the metabolic rate in the acid-soluble fractions from the lobus anterior of the hypophysis behaves like the corresponding fractions from the plexus. This proves that the change in the lobus anterior is also brought about by the parenchyma of the organ and not by the blood occurring therein.

About 25 % of the radioactivity is present in form of free phosphate, the rest in form of phosphate esters. The same values we also obtain for the epiphysis and lobus post. hypoph.

As regards the parts of the brain with lower activities, we may mention that the metabolism in the anterior part of tuber cinereum seems to be somewhat more powerful than that in the other parts. There are particularly many vegetative centres in this part of the brain. According to certain authors, there are special cells in this region with a glandular character (SCHARRER, 1940). These cells are situated particularly in the nucleus supraopticus and nucleus paraventricularis and may possibly produce hormones. These two circumstances may perhaps explain the somewhat livelier metabolism.

The metabolism of the lobus olfactorius is also comparatively high, which is not very surprising, since these animals have a well developed sense of smell.

Finally, we may mention that the substantia perforata generally shows a low metabolism. In a few sporadic cases the values are considerably higher and reach an order that is otherwise found only in the most active parts. This cannot as yet be explained. The variations in the metabolism of the substantia perforata do

Table 6.

*Relative specific activities of different brain parts and blood from three rats, weighing between 89 and 96 g., and an adult rabbit.
Specific activity of cerebellum = 100.*

Parts	Rats	Rabbit
Epiphysis	1465	3060
Lobus anterior hypophysis	545	2910
Lobus posterior hypophysis	650	2540
Plexus chorioideus	328	4560
Lobus olfactarius	88	188
Remainder of cerebrum	41	89
Thalamus	29	41
Anterior part of tuber cinereum	94	244
Posterior part of tuber cinereum	93	225
Corpus mamillare	87	144
Substantia perforata	248	131
Corpora quadrigemina	48	59
Cerebellum	100	100
Pons	36	66
Medulla	63	66
Blood	3640	9000

not follow alterations in the radioactivity of the blood; it is thus hardly probable that the admixture of blood explains the high values observed. Another possible explanation would be given by the small size of the pieces. The phosphorus content is also low, and therefore the analyses are somewhat unsure. Comparison with the lobus posterior, which contains about the same quantity of phosphorous, shows that that part does not show such marked variations. The variations in the metabolism of the substantia perforata are therefore probably caused by functional relationships.

In order to demonstrate that different metabolisms in different parts of the brain are not only found in fully developed rats, we also tested the brains of rats weighing between 80 and 90 g. As shown in Table 6, the same results were obtained as with fully developed animals. Analogous results were obtained in the study of a rabbit brain. In the latter case, the metabolism of the epiphysis was also very high, being at about the same level as that of the hypophysis. This shows that the powerful phosphate turnover in the epiphysis is not specific for the rat.

Summary.

This investigation constitutes an attempt to divide the brain into anatomically well defined regions and to compare the phosphate metabolisms of the latter by means of radioactive P.

(1) The highest phosphate content expressed in per cent of wet weight was found in the epiphysis, the lobus anterior and posterior of the hypophysis, the plexus chorioideus and the substantia perforata.

(2). Measurements with radioactive phosphorus suggested that a division of the parts of the brain into two groups was possible, viz. an active group with a lively phosphate metabolism and a less active group with a slow phosphate metabolism. The former group includes the epiphysis, the lobus anterior and posterior of the hypophysis and the plexus chorioideus, whereas the latter consists of the lobi olfactorius, parietalis and occipitalis, the thalamus, the anterior part of tuber cinereum, the posterior part of tuber cinereum, the corpus mamillare, the cerebellum, the pons, the corpora quadrigemina and the medulla oblongata. The substantia perforata has an intermediate position, but seems in the majority of cases to belong to the group with a lower phosphate metabolism. The activities were measured in relation to that of the cerebellum, which was taken as 100.

(3) The radioactive phosphorus accumulates 40 minutes after injection in the epiphysis to about 65 % in form of acidsoluble phosphate esters, in about 25 % in form of free phosphate and in about 10 % in compounds insoluble in trichloracetic acid.

(4) The activity of the epiphysis is generally higher than that of the hypophysis. The phosphate metabolism in this part was often twice or three times as vigorous as that of the lobus anterior hypophysis. It is therefore most probable that the epiphysis has an important function. Experiments are being carried out to investigate the function of this organ by the method described in this paper.

(5) The most active brain components, following immediately after the epiphysis, are the plexus chorioideus and the lobus anterior and posterior of the hypophysis. Owing to the considerable quantities of blood contained in the plexus chorioideus, particular conditions prevail in that organ. Those conditions are discussed

in some detail. The average phosphate metabolism of these parts of the brain is four to five times that of the cerebellum.

(6) Other parts of the brain tested exhibit, generally speaking, the same metabolism as the cerebellum. It seems to be significant that the anterior part of the tuber cinereum exhibits a somewhat livelier activity than the majority of the brain components belonging to this group.

We owe thanks to Docent J. MÖLLERSTRÖM for having placed his laboratory facilities at our disposal and for the interest he paid to our investigations and to Prof. MANNE SIEGBAHN for having provided us with the radioactive phosphorus necessary for this work.

Literature.

BARGMANN, W., Handb. Mikroskop. Anat. Mensch. 1943. VI: 4. 314—320.
BERBLINGER, W., Neue Dtsch. Klin. 1932. 10. 790.
BRIGGS, A. P., J. Biol. Chem. 1922. 53. 13.
CASPERSSON, T., Skand. Arch. Physiol. 1936. Suppl. 73.
HEVESY, G., Ann. Rev. Biochem. 1940. 9. 641.
HEVESY, G., and L. HAHN, Kungl. Dansk. Vidensk. selskap Biol. Med. 1940. 15. 7.
HEVESY, G., and J. OTTESEN, Acta Physiol. Scand. 1943. 5. 237.
HYDÉN, H., Acta Physiol. Scand. 1943. 6. Suppl. XVII. Diss.
KREBS, H. A., and H. ROSENHAGEN, Z. ges. Neurol. Psychiat. 1931. 134. 643.
LEVY, H., Acta Physiol. Scand. 1941. 2. 310.
MÖLLERSTRÖM, J., Acta Med. Scand. 1943. Suppl. CXLVII. 427.
SCHARRER, E., and B. SCHARRER, Ass. Res. Nerv. Ment. Dis. 1940. 20. 170.

From the Physiological Institute, The University, Uppsala.

Electrical Impedance Properties of Surviving Gastric Mucosa of the Frog.

By

TORSTEN TEORELL and RICHARD WERSÄLL.

Received 10 July 1945.

In connection with some investigations in this laboratory on the formation of hydrochloric acid by the isolated, surviving gastric mucosa of the frog an interest arose as to the applicability of electrical conductivity measurements as a mean of recording changes in the electrolyte permeability.

Measurements of the electrical conductivity employing alternating current of varying frequency have been used frequently as a method of characterizing cell and tissue permeability ("the electrical impedance method"). The fundamental ideas were developed in particular by GILDEMEISTER, PHILLIPSON, OSTERHOUT, McCLENDON, LULLIES, FRICKE and COLE. A great variety of biological objects have been studied. Some more recent works of interest in this connection are the papers by GERSTNER and by HOFMANN on frog skin and by ROSENDAL (1940) and TRIER (1943) dealing with the human organism (skin). Especially related to our problem are PLÜGGES experiments on the polarization capacity of the gastric mucosa of the frog.

A closer study of the previous literature from our point of view revealed so many imperfections that it was thought worth while performing some new experiments with an improved technique. The results of these experiments will be given below.

*Principles of the impedance method:*¹ A brief outline of the GILDEMEISTER-LULLIES-COLE way of characterizing tissue impedance is necessary for the discussion of the results.

¹ For a fuller treatment of the theory of alternating current measurements on biological objects refer to the reviews by COLE (1933, 1940), LULLIES (1930), von MURALT (1935) and DUYFF (1942).

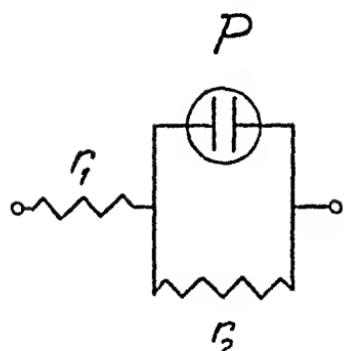


Figure 1. The electrical equivalent scheme of cells or tissues.
— r_1 = resistance of ion permeable structures inside and outside of the cells. r_2 = membrane "parallel" resistance corresponding to membrane ion permeability. — P = a "polarization element" corresponding to ion impermeable structures.

Any tissue may be approximately represented by an electrical equivalent circuit according to Fig. 1. The resistances r_1 and r_2 are pure ohmic resistances and P is a "polarization capacity" or "polarization element" (COLE), in main due to "diffusion capacity" and "double layer capacity".

The effective resistance of this circuit, the impedance (Z), can always be resolved into two components, an ohmic resistance (Wirkwiderstand) R and a capacitive reactance (Blindwiderstand) X , expressable in ohms (Ω). R and X can either be considered as lying in series (R_s and X_s) or in parallel (R_p and X_p). The interrelations between these quantities are geometrically represented by the diagram, Fig. 2. The significance of the phase-

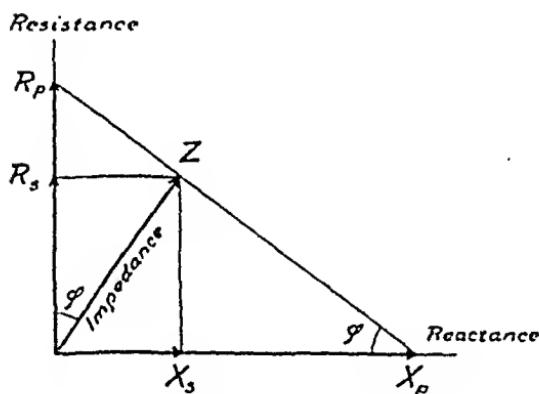


Figure 2. Vector diagram showing the interrelation between various impedance quantities. Z = total impedance. R_s , R_p = series or parallel resistance. X_s , X_p = series or parallel reactance. φ = the phase angle.

angle, φ , is also clear from the diagram as
— $\operatorname{tg} \varphi = \frac{R_p}{X_p} = \frac{X_s}{R_s}$.

Obviously the location of the terminal point of Z in the diagram can be fixed by either of the following three A. C. bridge methods, by determining at a given A. C. frequency 1) R_s and X_s (series resistance

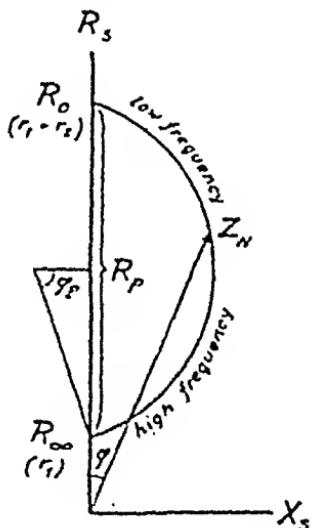


Figure 3. An impedance locus diagram. R_0 = zero frequency resistance, R_∞ = high frequency resistance. Z_N = impedance at the frequency N . φ = the phase angle at the frequency N . φ_P = the polarization angle (frequency independant). (r_1 and r_2 refer to the equivalent scheme of figure 1).

method), 2) R_p and X_p (parallel resistance method) or 3) Z and φ (the GRÜTMACHER method). If the frequency is varied, the vector Z will change both in magnitude and direction and its terminal will trace a curve of more or less semicircular form (= an "impedance locus diagram"), cf. Fig. 3.

The angle φ_p is the phase angle of the polarization element which we here will call the polarization angle. It is supposed to be independent of the frequency. In the special case that P is a pure capacity φ_p will become 90° and the center of the semi-circle located on the R axis. Besides by the polarization angle φ_p , the locus diagram is characterized by two important points: R_0 being the impedance at zero frequency (theoretically = D. C. resistance) and R_∞ the high frequency resistance, of the tissue. In terms of the equivalent circuit, figure 1, one obtains here $R_0 = r_1 + r_2$ and $R_\infty = r_1$.

As to the actual correspondence between these electrical quantities and the biological structures nothing definite can be said. The Gildemeister school has, as a rule, employed only one single frequency and interprets an increase of $\operatorname{tg} \varphi$, reactance or resistance as a sign of "Membrandichtung" and a decrease as a "Membranlockerung" (PLÜGGE *l. c.* p. 467). In other words, only one point on the circular arc locus diagram has been determined. Useful as this simple method may be for comparative purposes, it does not yield such full information as the method which measures at several different frequencies *i. e.* the impedance locus diagram method, where the result is expressed in terms of φ_p^1 , R_0 and R_∞ . According to current views one may roughly state that R_∞ corresponds to the resistance of the electrolytes inside and outside of the cells and $R_0 - R_\infty$ to the cell or tissue membrane resistance lying parallel as a shunt to the "capacity" P exhibited by these membranes. This parallel or shunt resistance $R_0 - R_\infty$ denoted R_p^1 therefore gives indication of permeability changes in such a direction that a decrease of this quantity may signify an increased "porosity" (Membranlockerung), or more strict, an increased electrolyte transfer. A change of φ_p yields information solely of the polarization element, *i. e.* the ion impermeable membrane structures (cf. COLE 1940) in such a manner that the larger φ_p the larger is the "polarizability" and the smaller the permeability of the structures containing P (cf. DIEHL p. 164).

¹ Note the difference between φ and φ_p , the former denotes the phase angle of the whole system of tissues including surrounding baths and electrodes, whereas φ_p is a characteristic of the polarization element freed from parallel or "loss resistances". The R_p of Fig. 2 and of Fig. 3 are not identical.

Methods.

1. The preparation and mounting of the gastric mucosa was performed according to DELRUE (1930) with the modifications introduced by GRAY and collaborators (1940). The isolated mucosa was mounted in a dual-chambered bath (Figure 4) between two flanges with a circular opening of 1 cm in diameter. The chamber which bathed the secretory side of the mucosa had a capacity of 3 ml and was generally filled with H_2O or 0.7 % NaCl solution. The other chamber contained

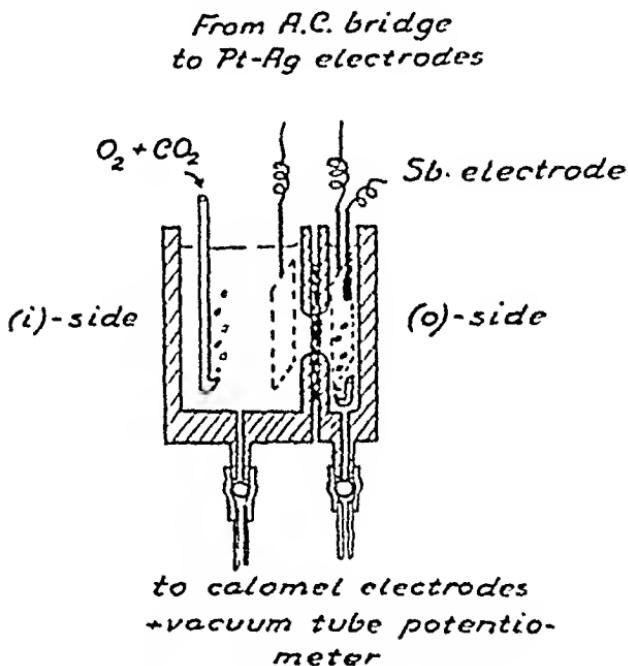


Figure 4. Scheme showing the perfusion apparatus. The mucosa preparation is inserted between the inside (i) and outside (o) chamber.

15 ml of an artificial nutrient solution (modified Ringer solution according to GRAY and coll.). Both chambers were aerated with 3 à 5 % CO₂ in pure O₂. The whole apparatus was made of Perspex glass and was square in form. The temperature was 20 ± 2 °C.

The leads for mucosa potential measurements were arranged according to Figure 4. The pH determinations were made by aid of a glass or antimony electrode inserted in the (o)-solution, the (o)-side calomel electrode being the reference electrode. Both electrodes were connected to the same vacuum tube potentiometer as used for the mucosa potential measurements.

2. *Impedance measurements.* The conductivity electrodes were 2×2.5 cm silver plates platinized according to JONES and BOLLINGER (some AgCl formed at the platinizing was dissolved in ammonia). These electrodes showed a negligible polarization over

the frequency range employed. They were inserted in a fixed position (by means of small grooves in the chamber walls) each time an impedance run was made, each electrode being 5 mm apart from the mucosa membrane. When H_2O had been used as (o)-side bath it was substituted by 0.7 % NaCl during the impedance run.

The A. C. bridge methods for the impedance determinations were the following 1) in the earlier experiments a "parallel resistance method" with a set up as schematically shown in Fig. 5. Here the unknown impedance was balanced and expressed by R_p and X_p ($= 1/\omega C_p$) (*vide supra*). Transformations into R_s and X_s could be made by aid of the conventional formulas (*cf. HAUGE*). 2) In more recent experiments the more convenient and time saving "GRÜTZMACHER method" was employed with a wiring according to Fig. 5. Here the impedance Z and the phase angle φ were read directly (*cf. GRÜTZMACHER's paper*).

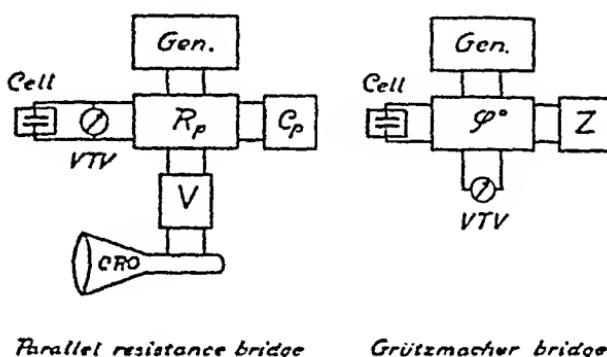


Figure 5. Block scheme showing the arrangement with the impedance measurements. Gen. = oscillator. V = amplifier. VTV = vacuum tube voltmeter. CRO = cathode-ray oscillosgraph. R_p , C_p , φ° , Z = balancing devices.

The oscillator furnished frequencies from 0.01 to 13 kHz of a good sine wave form. The A. C. potential over the electrodes was low, 10 ± 2 mV at all frequencies. All usual precautions against errors due to capacity to earth etc. were taken. The accuracy of the methods was controlled on "dummies" composed of known resistances and condensers and was approximately $\pm 2\%$ at Z values $> 100 \Omega$, the angle φ could be determined to $\pm 0^\circ.5$ or less.

In the diagrams and tables presenting the results the resistance figures of the electrodes + the bath solutions have been subtracted (the reactance was negligible). These figures were determined in "blank" runs using a filter paper instead of the mucosa membrane. For 0.7 % NaCl the figure was $100-105 \Omega$ at 0.01 kHz depending on the temperature and a few ohms less at 13 kHz. A complete run including measurements at ten different frequencies took about 10 minutes.

3. *Mucosa potential measurements* were performed by aid of the two identical calomel electrodes connected to the (i)- and (o)-chamber. A direct reading vacuum tube potentiometer gave readings good to about ± 0.5 mV. The sign of the potential is that of the (o)-side (the convention adopted for the sign was the following: the potential genera-

ted across a filter paper membrane when diluted HCl was placed in the (i)-chamber and H_2O in the (o)-chamber was defined as positive on the (o)-side).

4. *The construction of the impedance locus* and measurements on the locus diagrams were performed graphically. By trial a circle was found out, which gave a satisfying fit to the points obtained at the different frequencies in a plot with series resistance R_s as ordinate and series reactance X_s as abscissa. Often a considerable departure from a circular arc was observed at the highest frequencies, these points were then disregarded at the circle construction. The reason for this departure is unknown, but has been reported in other papers on impedance measurements (*cf.* for instance COLE 1940 p. 116). Once the center had been obtained, it was an easy matter to find φ_p , R_p and R_∞ (*cf.* Figure 3).

Results.

The impedance characteristics of the gastric mucosa during different conditions are presented below as the actual locus diagrams or as values for φ_p , R_p and R_∞ obtained from the corresponding locus curves. In all experiments where no special notice is made the mucosa has been normal in the sense that a distinct acid secretion has been obtained, *i. e.* the pH of the (o)-chamber has decreased to 4 or generally less within a few hours. In most instances this acidification has taken place spontaneously, otherwise the addition to the (i)-chamber of 0.2 ml of a 0.1 % histamine hydrochloride solution sufficed to provoke an acid secretion.

The effects of the following conditions were especially studied:

- 1) spontaneous gastric acid formation
- 2) induced acid formation
- 3) mechanical treatment
- 4) cell poison (chloroform)
- 5) alternating current flow
- 6) specific cation effects (K^+ , Na^+ , H^+).

1. *Spontaneous gastric acid formation* was obtained in more than $\frac{2}{3}$ of all the investigated cases (more than one hundred animals) after a "latency time" of one half to one hour after the completion of the preparation. Table I shows the course of a typical experiment (*cf.* also Figure 8 between 0^{h00m}—4^{h00m}). During the latency time it is especially noticeable that the "shunt" resistance R_p starts with a very low value which increases rapidly with several hundred per cents and reaches a maximum after

Table I.

*Spontaneous gastric acid formation.
0.7% NaCl in the (o)-side chamber.*

Time	Treatment	pH (o)-side	φ_p°	R_p Ω	R_∞ Ω	(o) — (i) mV
0 ^h 00 ^m	Preparation finished . . .	6.07	—	—	—	— 4.4
05	Impedance run A	5.67	55	76	16	— 4.1
25	* * B	5.67	55	235	20	— 11.3
48	* * C	5.45	57	322	27	— 15.1
1 ^h 37 ^m	* * D	4.30	63	303	27	— 11.6
2 ^h 40 ^m	* * E	3.31	64	260	28	— 12.2
3 ^h 25 ^m	* * F	3.06	72	208	27	— 11.4
35	Changed NaCl in (o) . . .	—	—	—	—	—
36		4.19	—	—	—	— 11.8
4 ^h 23 ^m	Impedance run G	3.25	73	214	26	— 14.0
6 ^h 10 ^m	* * H	2.77	78	190	26	— 14.8
7 ^h 35 ^m	* * I	2.61	79	200	27	— 18.0

about an hour when the acid production has just started. Later on, it remains more or less constant, even substitution of the acidified (o)-solution against fresh, neutral saline causes but a slight change. — The polarization angle φ_p increases in general slowly during the experiment without any "breaks" relating to the acid formation. — The high frequency resistance R_∞ remains unchanged within the limits of the experimental errors. — The "membrane potential" across the stomach preparation has a typical course: it starts with almost zero potential but shifts towards negative values (up to -5 à -20 mV) simultaneously with the beginning acid production and the rise of R_p .

2. *Induced gastric acid formation* is demonstrated in Table II. In this case for some unknown reason no spontaneous acid secretion took place. Here both R_p and the membrane potential remained at low values. A marked "spike" of R_p was, however, observed when acid started to come after the histamine addition. This case is typical for a "bad" stomach preparation: none or a very weak spontaneous acid formation and low figures for the electrical parameters.

3. *Mechanical treatment:* In general the stomach preparation was rather insensitive towards mechanical treatment as mild scratching on its surface when fixed in the apparatus. In several cases one could successfully remove the top layer of the mucosa cell layer. In spite of this operation the acid secretion continued with unchanged intensity. The A. C. impedance parameters,

Table II.

*Non-secreting mucosa where histamine induced acid formation.
0.7 % NaCl in the (o)-side chamber.*

Time	Treatment	pH (o)-side	φ_p [°]	R_p Ω	R_m Ω	(o) — (i) mV
0h00m	Preparation finished ...	7.25	—	—	—	+ 0.5
10	Impedance run A	6.43	48	38	12	+ 0.8
38	• • B	5.95	50	48	17	- 1.1
1h05m	• • C	6.05	50	66	24	- 0.3
1h20m	• • D	6.03	49	94	26	- 3.5
2h25m	• • E	5.95	49	116	26	- 5.2
2h31m	Changed NaCl in (o) ...	—	—	—	—	—
2h32m		6.22	—	—	—	- 5.3
3h12m	Impedance run F	5.20	51	116	26	- 6.1
3h55m	• • G	5.20	50	118	26	- 7.1
4h08m	Histamine added in (i) ..	—	—	—	—	—
4h39m	Impedance run H	4.39	40	180	21	- 7.3
4h48m	Changed NaCl in (o) ...	—	—	—	—	—
5h45m	Impedance run I	3.77	49	132	25	- 7.3
8h25m	• • K	2.85	55	114	23	- 7.0
22h23m	• • L	5.35	62	67	4	- 6.4

however, were "shocked" in the sense that both the shunt resistance R_p and the polarization angle φ_p suddenly decreased markedly after the operation (Figure 6 a, curve C before and D immediately after the injury).

There is also evidence of a good "recovery", a few hours later both R_p and φ_p have again risen considerably (cf. curve E and F).

4. Cell poison. Both KCN and chloroform added to the nutrient solution killed the stomach preparation when present in sufficient amounts. Here a case of chloroform poisoning is reproduced in Figure 6 b. The "killing effect" as reflected by the impedance characteristics is very typical. The impedance locus arcs "shrink" enormously and disappear finally. It is the R_p which vanishes, the polariza-

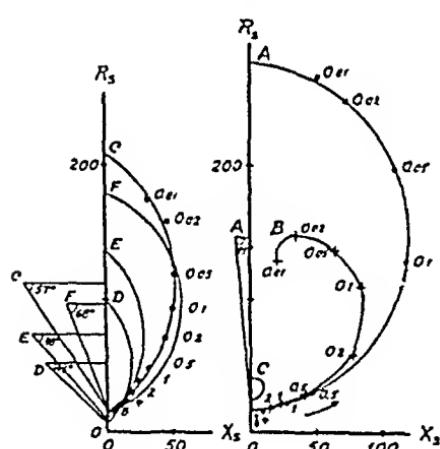


Figure 6. a) Left part: Locus diagram on the effect of a mucosa operation. C before, D immediately after the operation, E = 1 hour, F = 2 hours after the operation. b) Right part: Locus diagram on the effect of chloroform poisoning. A = before addition of CH_3Cl (the mucosa was treated with n/10 KCl on the (o)-side). B = 10^m after, C = 1^h after the CH_3Cl addition. (Figures on the arcs in kHz.)

tion angle seems to be less affected. The membrane potential also decreases. The "killing" process can extend over several hours if the poisonous dose is small. The effect of KCN was rather similar to that of the chloroform.

5. *Alternating current flow* is obviously an important factor to know something about in this study. Most authors employing the A. C. conductivity method on living objects have avoided strong currents. Especially GERSTNER (1939 p. 602) has made a systematical investigation as to the influence of the current density on frog skin polarization and recommends an A. C. voltage < 0.1 V (at 1 cm^2 skin area) in order to obtain capacity and resistance values which are independent of voltage and time. The experiences with frog stomach preparations made here are in agreement with GERSTNER's statements. In Figure 7 some locus diagrams are shown which demonstrate the injurious effect of a high voltage (curve C was measured with 1 V as compared with curve A and B which were exposed to 0.01 respectively 0.1 V). There is an immediate "shrinkage" of the locus arc similar to the chloroform effect. Here, however, a tendency to a recovery was visible provided the exposure to the higher voltage was not too long (*cf.* curve D at 0.01 V).

6. *Ionic effects* can be demonstrated by changing the composition of the solutions on both sides of the stomach preparation. Only the influence of substituting sodium for potassium (or hydrogen) ions on the outside (*i. e.* the side which secretes the gastric HCl) will be discussed here. Not only in the natural condition, but also in the excised, surviving state is this part of the stomach tissue insensitive towards the ionic composition of the solution bathing it. Even pure water does not seem to injure the mucosa. Unfortunately there arise several experimental difficulties due to the very low conductivity when attempting

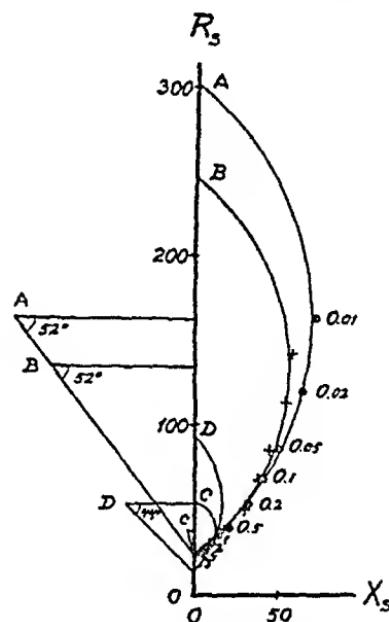


Figure 7. Locus diagram on the effect of alternating current flow. A = 0.01 V, B = 0.1 V, C = 1 V. (The exposures lasted 5 to 10 minutes, the intervals 5 min.)

to measure the A. C. impedance with pure water in the (o)-side. Therefore, all measurements were made either with "physiological" saline (0.7 % NaCl) constantly present or with this solution as a substitute for water during the periods of the impedance runs. No conclusive differences between the impedance effects of pure water as compared with saline could be detected, the sa-

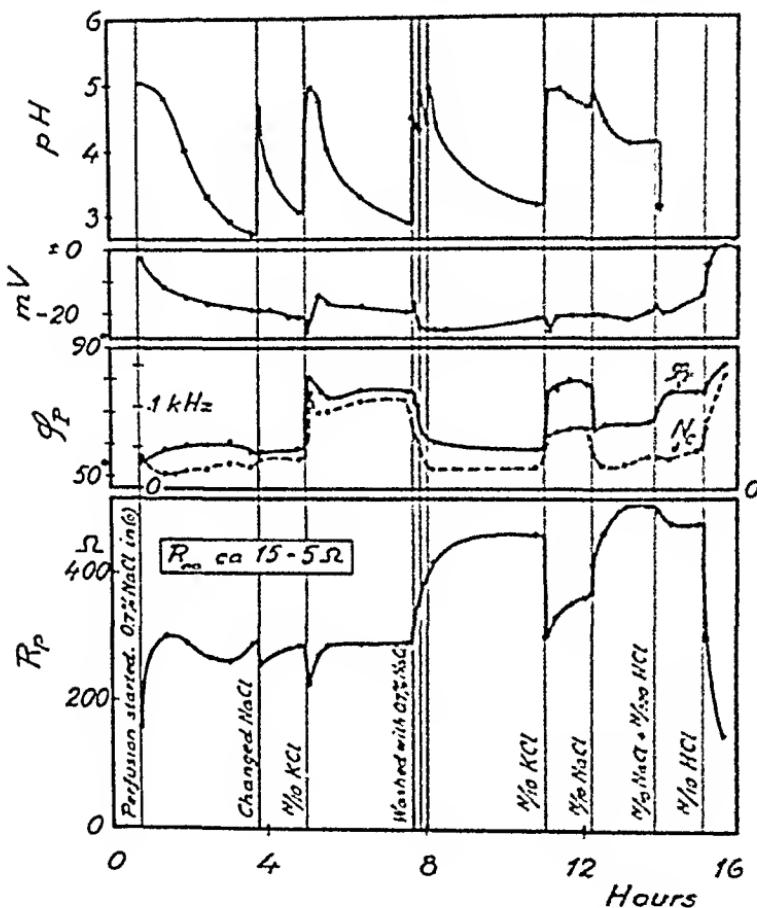


Figure 8. Diagrams on an experiment with spontaneous gastric HCl-secretion where the (o)-side solution was changed from NaCl to KCl. Comments in the text.

line results were, however, more reproducible and were therefore mostly resorted to.

Some very marked effects were constantly observed when NaCl was substituted for N/10 KCl as is evident from the experiment shown in Figure 8. In the first place it is to be noted that this substitute has no influence on the ability of the mucosa to secrete acid as can be seen by examining the pH curve. As regards the *mucosa potential* the only striking effect is a tem-

porary but slight increase of the negative potential a short period after the KCl additions.

The most striking effect, however, is noticed on the polarization angle φ_p . KCl changes φ_p rapidly towards higher values, sometimes near 90° , which means that the locus arcs appear as good semicircles (curve A in Figure 6 b is actually a "potassium effect" and should be compared with, for instance, curve C in Figure 6 a which is a typical NaCl locus arc). This "potassium effect" on φ_p is markedly reversible: φ_p is high with KCl and decreases if NaCl is again used as the (o)-solution (*cf.* Figure 8 between 5^h00^m and 12^h05^m).

The "potassium effect" on the parallel resistance R_p is also very apparent but rather complicated: The general trend seems to be that KCl tends to decrease R_p when NaCl may increase it, at least when these solutions are used consecutively (in Figure 8 there is, for instance, the temporary decrease after the KCl additions at 5^h00^m and 11^h00^m and the rather sharp rises of R_p at 7^h50^m and 12^h05^m when NaCl was added). On the whole it appears as R_p and φ_p vary inversely under the influence of KCl or NaCl added to the (o)-side.

No certain effects on the high frequency resistance R_∞ were observed when exchanging NaCl for KCl or HCl.

The effect of hydrogen ions seems to be somewhat similar to the K-effect but is often erratic (*cf.* 13^h55^m and 15^h00^m).

Discussion.

The experiments on the isolated, surviving mucous membrane from the frogs' stomach have clearly demonstrated that the A. C. conductivity varies with the viability of the preparation and after mechanical, electrical or chemical treatment. The main observations will now be discussed.

1) The mucosa membrane had a small value on the parallel resistance R_p after the preparation, after rough mechanical treatment, after poisoning with CH_3Cl and after electrical injury with high alternating current density. This would signify that the "porous" cell or tissue membrane permeability towards electrolytes becomes high during these circumstances (*cf.* p. 245). Such a fact would fit well with the postulation of a low or lowered viability of the stomach preparation under the conditions mentioned. PLÜGGE draws similar conclusions as regards narcotica.

GERSTNER (1941) has analytically shown that the permeability of sulphocyanate ions (SCN^-) actually runs parallel with a decrease in the "Blindwiderstand" at a given frequency and seems to assume that these ions penetrate through the intercellular route (i. e. Fig. 1 on p. 8, where it is possible to see that a quantity corresponding to R_p in the sense used here, also diminishes when the permeability rises).

If one grants the supposition that R_p is a measure of the cell and tissue membrane electrolyte transport one may be inclined to explain the so called "*potassium effect*" observed above as an indication that the cells, or whatever tissue element may be concerned, have a high permeability for KCl . $NaCl$ which tends to increase R_p after KCl treatment would then be considered as less permeable. The same would apply to HCl which generally also increases the R_p , at least when this acid is formed naturally.

2) The behaviour of the polarization angle is not so easy to explain in terms of permeability. It is obvious from our experiments on frog stomach mucosa that φ_P reacts¹ very promptly towards the exchange $Na^+ \rightarrow K^+$ and the reverse but is less sensitive to mechanical stimulation or a narcotic as CH_3Cl . According to the interpretation favoured by, for instance DIEHL, an increased polarizability, marked by a high value of φ_P , would indicate a reduced permeability of the cell membranes proper. According to COLE (1940) the membrane capacity giving rise to φ_P would represent the ion impermeable structures. Hence, the observed φ_P variations would signify some changes of these structures.

GERSTNER on the other hand employs the series capacity C_s at one low frequency as a permeability indicator and demonstrates that this quantity varies with the magnitude of the SCN^- transport through frog skin. COLE-DIEHL's and GERSTNER's methods are not directly comparable because it may be theoretically possible that the φ_P as defined here and by COLE and DIEHL can remain constant in spite of a variation of the C_s . In fact, a system corresponding to any frequency independent capacity and a constant parallel resistance like Figure 1 may always yield the same locus diagram regardless of the size of the capacity. The difference will solely consist in a different position on the locus arc of any one point corresponding to a given frequency (which is easy to understand by considering the definition of the $-tg\varphi$ as equal to $1/R_s\omega C_s$). If the same situation would occur

¹ This seems to be an unusual event, because COLE (1940) emphasizes that the membrane resistance under experimental conditions is more easily subject to changes than the membrane capacity which in general remains relatively unchanged. GUTTMAN made the same observation on frog sartorius muscle during the action of chloroform.

to a frequency dependent capacity as is generally thought to exist in the "polarization elements" of the living tissues, it would necessitate also some kind of definition of this frequency dependence. An attempt at such a definition is made by COLE who introduces the "characteristic frequency", (N_c), i. e., the frequency corresponding to the maximum of the series reactance X_s . In Figure 8 the dotted line in the section showing φ_p denotes the observed N_c variation after the treatment with NaCl and KCl-solutions. It is evident that this quantity as well as the polarization angle φ_p is markedly influenced by the ionic environment. In fact, the changes of N_c reflect the exchange $KCl \rightleftharpoons NaCl$ even better than φ_p and R_p . On the whole there is a good parallelism between the two first mentioned quantities.

Summarizing, we may state that our observations seem to indicate that the "polarization elements", probably located in the cell membranes, are subject to ionic influences in direction of a low potassium permeability of the *cell membranes*, whereas the changes of R_p on the contrary would indicate an increased *intercellular* potassium transport as compared with sodium. We believe, however, that such an interpretation must be taken with extreme caution at least as regards the significance of changes of φ_p and N_c .

As yet, the only A. C. quantity which seems to possess an unambiguous significance in the GILDEMEISTER-COLE scheme is the "parallel resistance" R_p , it appears to be a good (inverse) measure of the ionic tissue permeability. The exact biological meaning of other characteristics as the polarization angle will not be clear until the general nature of membrane polarization is more elucidated. We agree with GERSTNER, COLE and others that model experiments on well defined, non-living membranes are highly desirable.¹

In expectation of a better physical ground for the understanding of polarization phenomena one has, however, to be content with the fact that A. C. impedance quantities may be useful measures on alterations in living cells or tissues, alterations caused by some kind of stimulation or injury, it may be of an electrical, mechanical or chemical nature (*cf.* for instance COLE & CURTIS' studies on the transverse A. C. conductance of the electrically stimulated *LOLIGO* nerve or HOFMANN's experiments with narcotics and analeptics of frog skin etc.).

3) As regards the behaviour of the *mucosa membrane potential* it seems premature to attempt any discussion. PLÜGGE's state-

¹ We have studied moisture proof cellophane in a number of preliminary experiments. It has shown A. C. impedance properties which in several respects resembles the living membranes.

ment that there exists a "Parallelgehen" between polarization capacity and mucosa potential can neither be confirmed nor denied with the data at hand. Some interrelation may perhaps exist.

This work has been aided by grants from the Rockefeller Foundation and the Andersson Foundation. Our thanks are also due to Miss INGRID AHRNE for skilful technical assistance.

Summary.

The alternating current impedance of the isolated, surviving stomach mucosa from frog was examined over the frequency range 10—13,000 c/s. The results were expressed in terms of impedance locus diagrams according to GILDEMEISTER-LULLIES-COLE.

In general it was found that different states of activity and external conditions had a marked influence on the impedance quantities such as the "parallel resistance" and the "polarization angle". On the whole, it was found that strong stimulation (in connection with secretion, or electrical current flow) or injury (mechanical or chemical) in particular affected the parallel resistance.

Impedance quantities related to the polarization properties of the mucous membrane, *i. e.* the "polarization angle" and the "characteristic frequency", were sensitive to the exchange of sodium ions against potassium ions ("potassium effect").

The observations are discussed according to some current hypotheses. It is emphasized that great caution must be exercised when attempting to interpret the impedance quantities in terms of cell and tissue permeability.

References.

COLE, K. S., Cold Spring Harbor Symp. 1933. *1.* 107.
COLE, K. S. and H. J. CURTIS, Cold Spring Harbor Symp. 1936. *4.* 73.
COLE, K. S., and H. J. CURTIS, *J. gen. physiol.* 1938—39. *22.* 649.
COLE, K. S., Cold Spring Harbor Symp. 1940. *8.* 110.
COLE, K. S., *J. gen. Physiol.* 1941. *25.* 29.
DELRIE, G., *Arch. inter. Physiol.* 1930. *33.* 196; 1933. *36.* 129;
1934. *38.* 126.
DIEHL, F., *Z. ges. exp. Med.*, 1937. *100.* 145.

DUYFF, J. W., Low-frequency impedance of animal tissue. Amsterdam 1942.

FRICKE, H., Cold Spring Harbor Symp. 1933. 1. 117.

GERSTNER, H., Pflüg. Arch. ges. Physiol. 1939. 242. 113.

GERSTNER, H., Pflüg. Arch. ges. Physiol. 1939. 242. 587.

GERSTNER, H., Pflüg. Arch. ges. Physiol. 1940—41. 244. 68.

GILDEMEISTER, M., Pflüg. Arch. ges. Physiol. 1919. 176. 84.

GRAY, J. S., J. L. ADKISON and K. ZELLE, Amer. J. Physiol. 1940. 130. 327.

GRAY, J. S., and J. L. ADKISON, Amer. J. Physiol. 1941. 134. 27.

GRÜTZMACHER, M., Telegraphen- u. Fernsprech-Technik 1934. 23. 27.

GUTTMAN, R., J. gen. Physiol. 1939. 22. 567.

HAUGE, B., Alternating current bridge methods. London 1932.

HOFMANN, H., Arch. exp. Path. Pharmak. 1943. 201. 529.

JONES, G., and G. M. BOLLINGER, J. Amer. Chem. Soc. 1935. 57. 281.

ULLIES, H., Abderhaldens Handb. der biol. Arbeitsmeth. V, 5 A, S. 1171.

ULLIES, H., Pflüg. Arch. ges. Physiol. 1929. 221. 296.

ULLIES, H., Pflüg. Arch. ges. Physiol. 1930. 225. 69.

MCCLENDON, J. F., Protoplasma 1927. 3. 71.

v. MURALT, A., Ergebni. Physiol. 1935. 37. 489.

OSTERHOUT, W. J. V., Injury, recovery and death, in relation to conductivity and permeability. Philadelphia 1922.

PHILIPPSON, M., C. R. Soc. Biol. Paris 1920. 83. 1399; 1924. 90. 373.

PHILIPPSON, M., Bull. Cl. Sci. Ac. Roy. Belg., 1921. 7. 387.

PLÜGGE, H., Pflüg. Arch. ges. Physiol. 1933. 232. 466.

ROSENDAL, T., The conducting properties of the human organism to alternating current. Diss. Copenhagen 1940.

TRIER, M., Kliniske undersøgelser over det menneskelige legemes elektriske ledningsevne ved sygdomme i skjoldbruskkörtlen. Diss. Copenhagen 1943.

From the Institute of Medical Anatomy and the Institute
of Theoretical Physics, University of Copenhagen.

Studies on the Lymphocyte Production.

Investigations on the Nucleic Acid Turnover in the Lymphoid Organs.

By

ERIK ANDREASEN and JENS OTTESEN.

Received 3 August 1945.

Chemically the mitosis is chiefly characterized by changes in the nucleic acid content, large quantities of deoxyribose nucleic acid being concentrated in the karyoplasm during the mitosis (BRACHET, CASPERSSON a. o.). Consequently it may be possible, by measurements of the nucleic acid turnover, to obtain an estimate of the mitotic activity in a selected organ. The correctness of this view has been confirmed by investigations by v. EULER and HEVESY (1944) and ANDREASEN and OTTESEN (1944).

We have tried to answer the question about the importance of the different lymphoid organs as a source of blood lymphocytes by determining the rate of deoxyribose nucleic acid formation in various lymphatic tissues.

The lymphoid tissue atrophies with age and this atrophy is proportionally more pronounced than the involution of any other tissue in the organism. It would be reasonable to assume, therefore, that the magnitude of lymphocyte production is also subjected to regressive changes with increasing age. In this study we have consequently examined the nucleic acid turnover in three different age groups; one in which the rate of growth of the lymphatic tissue is very great; one in which the tissue has reached its maximum development, and one, finally, in which the lymphatic tissue of the thymus and the lymph nodes has been atrophied for a long time.

Material and Methods.

We have used albino rats of which the youngest group comprises infantile animals (3—5 weeks old). The next age group is made up of young mature animals (3—4 months old); the last group are old animals (1—2 years old). The development of the lymphoid tissue in each age group can be read from Fig. 1 which illustrates the growth and involution of the lymphoid tissue, estimated by quantitative determinations of the phosphorus content of the ribose- and desoxyribose nucleic acid in the individual lymphoid organs.

The rate of formation of nucleic acid was investigated by means of a radioactive tracer substance. The principle of the method may be summarized as follows: after injection of radioactive phosphorus (^{32}P) into an animal, it is possible shortly afterwards to trace the labelled phosphorus in the blood plasma and in various tissues of the organism. Here the labelled phosphate takes part in the various chemical reactions with the same probability as the other phosphate ions present. Thus, when nucleic acid molecules are built up, a certain fraction of the nucleic acid will be labelled with radioactive phosphorus. If the entire nucleic acid content of a lymphoid organ were formed within the experimental period, 1 mg nucleic acid P would contain the same percentage of ^{32}P as 1 mg phosphate P. If, on the other hand, 1 mg nucleic acid P has a lesser activity than 1 mg phosphate P — e. g., 1/100

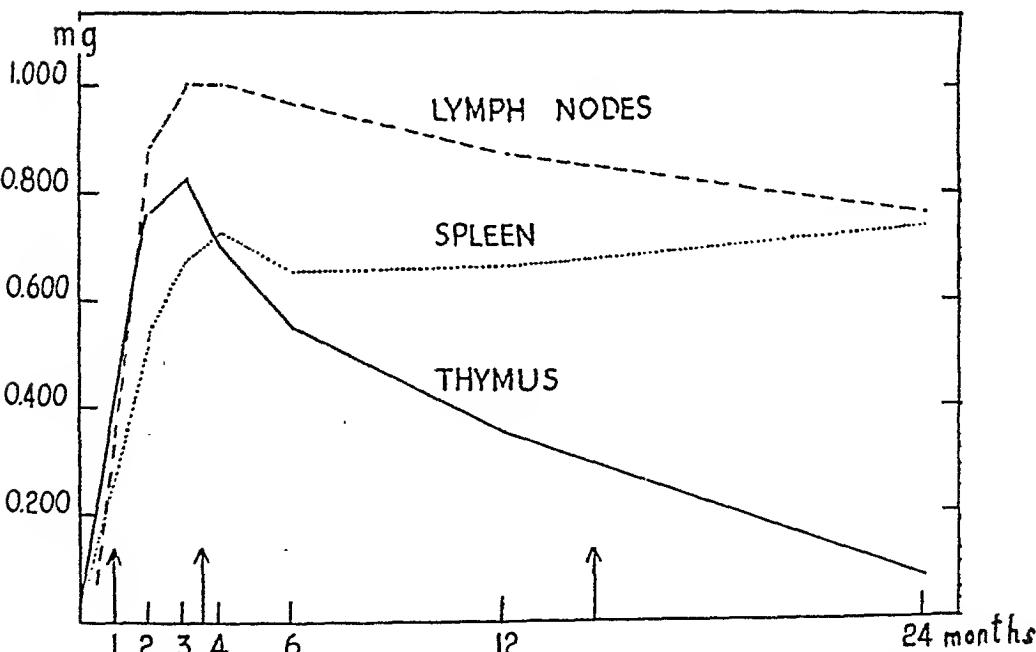


Fig. 1. Graphs showing averages for absolute amount of nuclein phosphorus in the lymphoid organs of female rats. The arrows indicate the stages of development at which the organs are examined in the present study.

of its activity — the amount of nucleic acid formed in the experimental period will make up only 1 per cent of the total nucleic acid content of the organ concerned. Assuming, of course, that phosphate P has the same activity throughout the experimental period.

For our experiments the labelled phosphorus was administered as sodium phosphate; it was dissolved in a physiological salt solution and injected subcutaneously in a quantity of 0.5—1 ccm per animal; the dose had an activity corresponding to about 1—4 microCurie, depending on the size of the animal. The rather low dosage in connection with the short experimental period probably excludes an effect of the β -rays, emitted by the active phosphorus on the formation of the labelled nucleic acid (see also p. 266).

Three hours after the injection the animal was killed by bleeding; a sample of blood was taken for determination of the activity of free plasma-P. The lymphoid organs were segregated, weighed and crushed in a mortar. In addition a thorough inspection of all organs was undertaken in order to be sure that the animal was perfectly normal. One animal, as a rule, was employed in each experiment. The youngest animals (20 days old), however, weighed only 30 grams each and in this case it was necessary on account of the method of extraction to pool the organs from 6 animals of the same litter.

The desoxyribose nucleic acid of the lymphoid organs was extracted and purified by a method described by KLEIN and BECK (1935) but adapted by HEVESY *et al.* (v. EULER and HEVESY, 1942; HEVESY and OTTESEN, 1943) to tissues containing radioactive phosphorus. The activity of the nucleic acid was measured by a Geiger tube counter; particulars of the method of measurement are given by AMBROSEN, MADSEN, OTTESEN, and ZERAHN (1945) and OTTESEN (1945).

Distribution of Injected ^{32}P in the "Free" Phosphate of the Lymphoid Organs.

The percentage ratio of the specific activity of nucleic acid P and the specific activity of the plasma phosphate P, extracted at the end of the experimental period, is often used as an expression for the nucleic acid turnover in an organ (specific activity denotes the percentage of the ^{32}P administered present in 1 mg P). Beside the nucleic acid turnover this ratio is also due partly to the different rate at which the labelled phosphate from blood plasma penetrates into the cells where nucleic acid molecules are built up. A comparison from organ to organ of this ratio therefore will be permissible only when the rate of penetration is the same. The uniform structure and vascularization of the lymphoid organs might justify the assumption that the rate at which the labelled phosphate penetrates the cell-membranes is about the

same in the different organs. This question is, however, of such great importance to the correct interpretation of our results, that we have determined the renewal of the "free" phosphate in the various organs of some animals belonging to different age groups. The free phosphate was extracted by 10 per cent trichlor-acetic acid and precipitated as ammonium magnesium phosphate; an aliquot of the solution was used for colorimetric P-determination ad modum Fiske-Subbarow-Teorell, the remaining fraction was used to measure the activity.

The results of the determinations of the "free" P extracted from different lymphoid organs and the plasma are seen in table 1

Table 1.

Distribution of Radioactive P in the "Free" Phosphate Extracted from Plasma and Lymphoid Organs.

Experimental Animals: Three Groups of Albino Rats (1—4 and 18 Months).

Organs	Percentage ratio of injected ^{32}P in 1 mg "free" P						
	I month		IV months			XVIII months	
	1 hour	3 hours	$\frac{1}{2}$ hour	$1\frac{1}{4}$ hour	3 hours	3 hours	3 hours
Thymus	3.21	1.88	0.99	2.04	1.81	1.50	1.04
Lymph nodes of intestines	2.02	1.14		1.59	1.22	1.10	1.12
Lymph nodes of skin	2.45	1.16	0.49	1.56	0.95	1.15	1.16
Spleen	1.95	1.44	0.68	1.40	1.33	1.55	1.08
Plasma	5.67	1.42	5.12		1.73	1.58	

The table shows that some time will pass before the plasma P comes into exchange equilibrium with the inorganic P of the tissues. In the thymus, the renewal takes place more quickly than in the other organs; this statement, however, does not concern the atrophied thymus (group XVIII); the figures show that the renewal in the different lymphoid organs from old animals is approximately identical. The difference in the rate of penetration of the injected ^{32}P is most easily understood, when the specific activity of the free phosphate is expressed in proportion to the specific activity of the phosphate of a single organ *e. g.* the thymus. This has been done in table 2 in which the renewal-rate in the thymus is taken as 100.

The rate of penetration thus changes with age. This confirms the observations by AHLSTRÖM, v. EULER and HEVESY (1944),

who found that phosphate ions penetrate considerably faster into the splenial cells of three days old rats than in the case of mature animals.

Table 2.

*The Relative Rate of Penetration of Radioactive P
into the Cells of the Lymphoid Organs.*

Organs	Percentage ratio of injected ^{32}P in 1 mg "free" P. The values of the thymus are taken = 100						
	I month		IV months			XVIII months	
	1 hour	3 hours	$\frac{1}{2}$ hour	$1\frac{1}{4}$ hour	3 hours	3 hours	3 hours
Thymus	100	100	100	100	100	100	100
Lymph nodes of intestines	63	61		78	70	74	109
Lymph nodes of skin	76	62	50	76	54	77	113
Spleen	61	76	69	69	76	105	108

We have not undertaken a correction for the presence of labelled phosphate in the extracellular tissue fluid. The amount of intracellular phosphate is so much larger than the contents of phosphate in the extracellular fluid (*conf. v. Euler, and Hevesy 1942*) that the specific activity of free organ-P depends essentially on the activity of intracellular P.

Specific Activity of Nucleic Acid Extracted from the Lymphoid Organs.

The measurements of the activity of nucleic acid extracted from the different lymphoid organs are recorded in tables 3—5. The tables contain statements of the specific activity of nucleic acid-P extracted from various organs together with data indicating the percentage ratio of the specific activity of free plasma phosphate extracted from the blood of the animals in question at the end of the experimental period (3 hours). The values obtained may be taken as an estimate of the nucleic acid turnover in the organs, as, in contrast to various acid-soluble phosphorus compounds, the deoxyribose nucleic acid molecules are renewed but very slowly (*v. Euler and Hevesy, 1942*). These figures do not indicate absolute values for the nucleic acid turnover. To obtain such values an average of the activity of "free" P during the whole experimental period would be required. We are, how-

ever, not so interested in the exact values for the nucleic acid turnover; we wish to examine only the ratio of the nucleic acid turnover in the different lymphoid organs, and for this purpose it will be sufficient to compare the activity of nucleic acid P determined at the end of the experimental period with the activity of "free" plasma P, also determined at the end of the experimental period.

Our results show that the nucleic acid turnover is considerably higher in the thymus than in the other lymphoid organs. Comparing the turnover in the different lymphoid organs it must be remembered (see above) that the rate at which phosphate ions penetrate into the thymic cells is somewhat greater than in the other lymphoid organs of the animals aged one and four months.

This means that the difference of the nucleic acid turnover in the thymus and the other lymphoid organs is somewhat lower than the values in tables 3 and 4 indicate.²

Compared with the nucleic acid turnover in other rat-organs (HEVESY and OTTESEN 1943; AHLSTRÖM, V. EULER and HEVESY 1944) the nucleic acid turnover in the lymphoid organs is very

Table 3.

*Desoxyribose Nucleic Acid Turnover of the Lymphoid Organs
in Infantile Animals.*

Age in days	Organs	Percentage ratio of injected amount of ^{32}P in 1 mg of nucleic acid P (Specific activity)	Percentage ratio of specific activity of nucleic acid and "free" P in plasma (Percentage renewal in 3 hours)
20	Thymus	0.031 ¹	8.5
	Lymph nodes of intestines	0.016 ¹	4.4
	Lymph nodes of skin	0.009 ¹	2.7
	Spleen	0.019 ¹	5.2
30	Thymus	0.190	6.5
	Lymph nodes of intestines	0.005	3.3
	Lymph nodes of skin	0.045	1.5
	Spleen	0.056	1.9
40	Thymus	0.070	7.8
	Lymph nodes of intestines	0.040	4.5
	Lymph nodes of skin	0.020	3.0

¹ These values refer to a litter of 6 animals, while the 30 and 40 days' figures refer to one rat only.

² When the difference in the rate of penetration is included in the calculations the nucleic acid renewal in the lymph nodes and spleen is about 50 per cent higher than indicated in tables 3 and 4.

Table 4.

Desoxyribose Nucleic Acid Turnover of the Lymphoid Organs in Young Mature Animals.

Age in months	Organs	Percentage ratio of injected amount of ^{32}P in 1 mg of nucleic acid (Specific activity)	Percentage ratio of specific activity of nucleic acid and "free" P in plasma (Percentage renewal in 3 hours)
4	Thymus	0.077	6.0
	Total of lymph nodes	0.022	1.7
	Spleen	0.034	2.7
	Bone marrow	0.130	10.0
4	Thymus	0.061	4.8
	Lymph nodes of skin	0.020	1.6
	Spleen	0.015	1.2
3	Thymus	0.0367 ¹	5.1
	Lymph nodes of intestines	0.0109 ¹	1.5
	Lymph nodes of skin	0.0081 ²	1.2
	Spleen	0.0069 ²	0.9
	Peyer's patches	0.0175 ¹	2.4

Table 5.

Desoxyribose Nucleic Acid Turnover of the Lymphoid Organs in Old Animals.

Age in months	Organs	Percentage ratio of injected amount of ^{32}P in 1 mg of nucleic acid (Specific activity)	Percentage ratio of specific activity of nucleic acid and free P in plasma (Percentage renewal in 3 hours)
12	Thymus	0.070	5.1
	Lymph nodes of intestines	0.011	0.8
	Lymph nodes of skin	0.010	0.8
	Spleen	0.010	0.8
17	Thymus	0.082	6.0
	Lymph nodes of intestines	0.017	1.3
	Lymph nodes of skin	0.018	1.3
	Spleen	0.009	0.7
	Bone marrow	0.170	13.0
14	Thymus	0.178 ²	5.8
	Lymph nodes of intestines	0.026 ²	0.9
	Lymph nodes of skin	0.032 ²	1.1

¹ These values apply to an experimental period of 2 hours.

² These values apply to an experimental period of 12 hours.

large. The only tissues examined so far in which the nucleic acid turnover was found to be of the same magnitude as in the thymus was the intestinal mucosa and the bone marrow. In our tables some figures are included indicating the nucleic acid turnover in the bone marrow; these figures are remarkable by being about twice the values of the thymus.

Nucleic Acid Turnover as a Measure of Lymphocyte Production.

It is on record that the desoxyribose nucleic acid turnover in various tissues is in some way dependent on the phenomenon of cell mitosis. Likewise, the desoxyribose nucleic acid turnover in an organ may be related to the mitotic activity of the organized tissues in question, provided that new-formed cells are not removed from the organ during the experimental period.

As regards the lymphoid organs, the values (see tables 3, 4 and 5) of nucleic acid turnover primarily denote the rate of lymphocyte production. Part of the new-formed nucleic acid molecules, however, is used to build up the tissue in general, and this process continues on account of the physiological wear and tear of the body. In the youngest age group growth takes place and in this case more nucleic acid molecules will be utilized. The question about the nucleic acid requirement during growth processes has been examined by v. EULER and HEVESY (1944); in a Jensen-sarcoma a quantity of nucleic acid used for growth of the sarcoma by 1 per cent is stated to amount to nearly 2 per cent of the total nucleic acid content in the sarcoma. Only about half of this quantity, however, is used in the formation of new tissue; the other half is used for renewal of "old" nucleic acid molecules.

In the lymphoid organs from the youngest of our experimental groups, we must face the fact that we measure the nucleic acid turnover in a rapidly growing tissue. During the 3rd and 4th weeks of postnatal life (*conf. ANDREASEN 1943*) the weight of the thymus and spleen are doubled. Those of the peripheral lymph nodes trebled, and the intestinal lymph nodes will even 5—6-double their weights. In the course of the experimental period (3 hours) we consequently must take into account an increase of the organ, which in the case of thymus and spleen will amount to about 1 per cent, as regards the peripheral lymph nodes 1.5 per cent, and in the case of the intestinal nodes 4—5 per

cent. We may assume that the organs originating from youngest litter (20 days old) have shown a growth-rate of this magnitude. This means that the tabulated figures for nucleic acid turnover in the mesenterial and peripheral lymph nodes are so small that they are swallowed up by the corrections due to the growth processes in the nodes; therefore there is nothing to indicate that a production of lymphocytes takes place. The difference in the nucleic acid turnover seen in the mesenterial and the peripheral lymph nodes (a difference not found in the older animals) may be explained by the different rate of growth presented by the two groups of lymph nodes at the time of examination. For thymus and spleen the magnitude of the nucleic acid turnover is so high that new-formed nucleic acid molecules must be used up for something else than the growth.

The other experimental animals in the 1 month's group were a little older (30 days and 40 days respectively) and the rate of growth of the organs already decreasing. The nucleic acid turnover in the different organs is still high. This, however, does not apply to the turnover in the spleen which are rather low being only a little higher than in the peripheral lymph nodes. Another circumstance must be considered, however, namely the size of the dose of radioactive phosphorus; into these two animals we injected a dose of about 2 microCurie which relatively correspond to the therapeutic dose employed by LAWRENCE *et al.* (1939) in humans suffering from lymphatic leucemia. These therapeutic applications extend over a long period and humans are probably more sensitive to radioactive phosphorus than rats, it is true, but it cannot be excluded a priori that the lower values in the above-mentioned two animals compared to youngest animals in which a similar dose was distributed over 6 animals, may be referred to a lowering of the mitotic rate on account of β -rays emitted from the radioactive phosphorus.

The magnitude of the physiological wear and tear in a tissue is defined by AHLSTRÖM, V. EULER and HEVESY, 1944; it is stated that the upper limit for the quantity per hour of new-formed nucleic acid in normal liver tissue amounts to 0.1 per cent of the total nucleic content. We are not able to give figures for the loss of nucleic acid owing to waste in the lymphoid tissue; but the values for liver tissue just mentioned are even being relatively high — has no influence on the high nucleic acid turnover found in the lymphoid organs.

In the lymphoid organs both emigration and immigration of lymphocytes are possible, and it is pertinent therefore to ask whether the new nucleic acid molecules are formed inside the lymphoid organs or whether they are brought on from other organs where the synthesis has taken place. A histological study of KINDRED (1940) throws some light upon this problem. By measurements of the mitotic rate in lymph nodes, spleen, thymus, and bone marrow in 15 and 20 days old rats, calculations showed that only the blood and the cortices of the lymph nodes take up small lymphocytes and that this supply is mostly furnished by the thymus. Furthermore KINDRED found that the spleen and bone marrow are able to produce a sufficient number of small lymphocytes to cover their intrinsic demand at this period of life.

Therefore the nucleic acid turnover in the thymus of infantile animals probably indicates a high lymphocyte production, while in the lymph nodes it is exclusively due to processes of growth which are followed by no lymphocyte production of any consequence. As to the spleen the interpretation of our studies is rendered difficult on account of the formation of red blood cells and granular leucocytes in the splenic pulpa being rather pronounced in the first month of life (JOLLY and ROSELLO, 1909; ANDREASEN, 1943).

Significance of the Various Lymphoid Organs in Relation to the Lymphocyte Production.

Assuming that the labelled deoxyribose nucleic acid found in an organ is also synthesized there, we can utilize the data in

Table 6.

Total Nucleic Phosphorus Content of the Lymphoid Organs in Female Albino Rats.

Age (months)	Thymus	Lymph nodes of intestines	Lymph nodes of skin	Spleen	Peyer's patches
new-born	0.024 mg			0.026 mg	
½	0.218 *			0.157 *	
1	0.431 *	0.155 mg	0.264 mg	0.262 *	0.067 mg
2	0.762 *	0.332 *	0.540 *	0.538 *	0.216 *
3	0.829 *	0.365 *	0.669 *	0.676 *	0.183 *
4	0.703 *	0.380 *	0.659 *	0.730 *	0.142 *
6	0.552 *	0.308 *	0.652 *	0.653 *	0.139 *
12	0.354 *	0.237 *	0.640 *	0.668 *	0.110 *
24	0.081 *	0.223 *	0.549 *	0.744 *	0.118 *

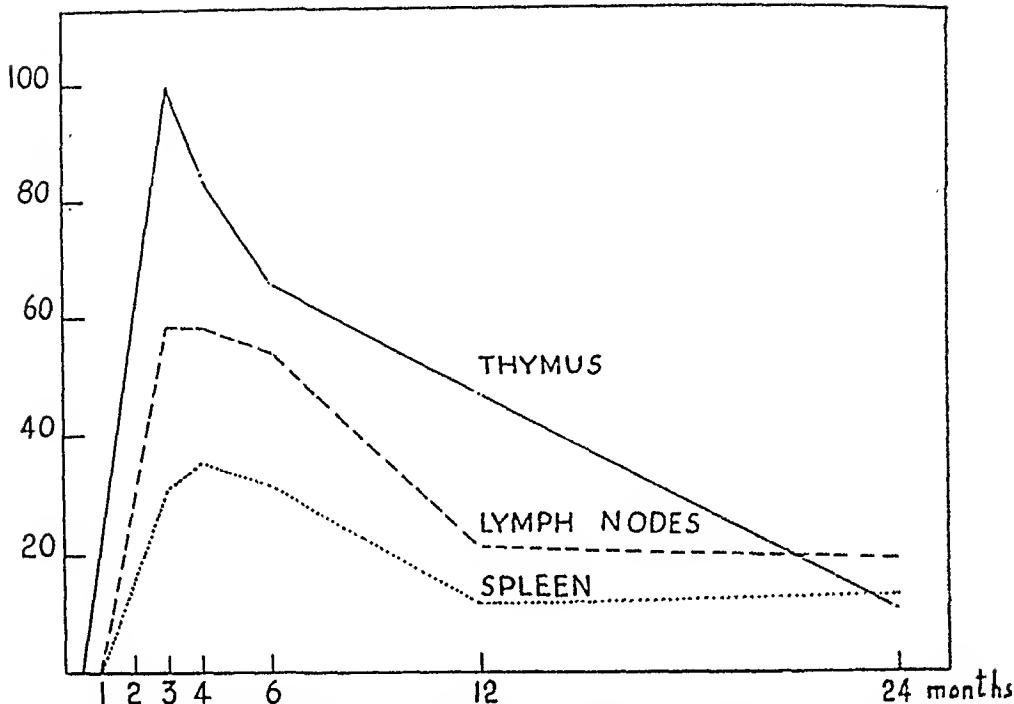


Fig. 2. Curves illustrating the possible conception of the relative extent of lymphocyte production in different lymphoid organs (conf. Text).

tables 3, 4 and 5 to obtain an estimate of the total amounts of desoxyribose nucleic acid synthesized in the various organs in a given period provided that the desoxyribose nucleic acid content of these organs is known. Now, we do not know the desoxyribose nucleic acid content of the organs in question, but data on the total nucleic acid content of such organs are available (see ANDREASEN, 1943); these figures are obtained by assuming that the residual phosphorus after the removal of the acid soluble phosphorus and the phosphatide phosphorus is nucleic acid phosphorus. The figures employed in the following calculations for the lymphocyte production in female animals, are given in table 6.

The uniform structure of the different lymphoid organs make us believe that the total nucleic acid and desoxyribose nucleic acid are present in fixed proportions. If this is the case we should be able to get an estimate for the content of the new-formed nucleic acid, meaning the rate of lymphocyte production, by multiplying the amounts of total nucleic acid with the percentage ratio of new-formed desoxyribose nucleic acid.

In Fig. 2 the result of such a calculation referring to female

animals is presented graphically. This diagram shows that the thymus is more important than lymph nodes and spleen for the lymphocyte production, a result which is in keeping with KINDRED's observations mentioned above. In our opinion the curves for the lymph nodes and the spleen represent the upper limits for the lymphocyte production since an immigration of lymphocytes — deduced from the scarcity of mitotic figures — probably takes place.

It goes without saying, that lymphocytes may be formed outside the lymphoid organ, in the diffuse lymphoid tissue, above all in the bone marrow. In this particular organ we have found the nucleic acid turnover to be rather high. Such a finding was to be expected in view of the fact that intense formation of granulocytes and erythrocytes takes place in the bone marrow.

Summary.

The importance of the various lymphoid organs as a source of blood lymphocytes is studied by determinations of the rate of desoxyribose nucleic acid formation in the organs under examination. The rate of formation of nucleic acid is estimated by means of the radioactive indicator method. The animal material is normal albino rats in different age groups.

A large nucleic acid turnover is observed in the lymphoid organs. The greatest renewal takes place in the thymus, the rate of renewal in mature animals being 5—6 per cent during the 3 hours' experimental period. The corresponding figures for the lymph nodes and the spleen are only 1—2 per cent.

Various considerations lead to the assumption that the desoxyribose nucleic acid turnover affords an estimate of the lymphocyte production in the different lymphoid organs. According to this conception, calculations show that the thymus must be the most important lymphocytopoietic organ except in old age when the weight of the organ is markedly reduced. Compared with the high thymic activity the other lymphoid organs possess only a slight lymphocytopoietic function.

We wish to express our sincere thanks to Professor G. HEVESY and Professor A. KROGH for kindly putting numerous facilities at our disposal.

References.

AHLSTRÖM, L., H. v. EULER, and G. v. HEVESY, Svenska Vet. Akad. Ark. Kemi, Mineral. Geol. 1944. 19 A. No 9.

AMBROSEN, J., B. MADSEN, J. OTTESEN, and K. ZERAHN, Acta physiol. Scand. (in press).

ANDREASEN, E., Acta path. microbiol. Scand. 1943. Suppl. 49.

ANDREASEN, E., and J. OTTESEN, Ibidem. 1944. Suppl. 54. 25.

v. EULER, H., and G. v. HEVESY, Danske Videnskab. Selskab, Biol. Medd. 1942. 17. No. 8.

v. EULER, H., and G. v. HEVESY, Svenska Vet. Akad. Ark. Kemi, Mineral. Geol. 1944. 17 A. No. 30.

HEVESY, G., and J. OTTESEN, Acta physiol. Scand. 1943. 5. 237.

JOLLY, J., and H. ROSELLO, C. R. Soc. Biol., Paris 1909. 66. 40.

KINDRED, J. E., Amer. J. Anat. 1940. 67. 99.

KLEIN, G., and J. BECK, Z. Krebsforsch. 1935. 42. 163.

LAWRENCE, J. H., K. G. SCOTT, and L. W. TUTTLE, Internat. Clin. 1939. 3. New Ser. II. 32.

OTTESEN, J., Acta physiol. Scand. (in press).

From the Institute of Normal Anatomy, Aarhus University, and
the Laboratory of Zoophysiology, Copenhagen University.

Studies on the Blood Supply to Certain Regions in the Lumbar Part of the Spinal Cord.

By

ERIK KROGH.¹

Received 9 August 1945.

In an earlier investigation (E. KROGH 1945) of the effect of acute ischemia on the cells of the anterior horn in the lumbar gray matter in the rabbit the following observation was made. The cells in the circumference of the horn appeared to be more resistant towards ischemia than the central ones. A somewhat similar observation was described by EINARSON and RINGSTED (1938). They found that normally the cells in the periphery of the anterior horn are more resistant against long continued lack of E vitamin than the cells near the center. EINARSON and RINGSTED were of opinion that this was due to a special innate power of resistance in the cells. EINARSON writes (p. 72) quoting BOK (1928) that cells innervating extensor and supinator muscles are preferentially situated in the periphery of the anterior horn while cells innervating adductor and flexor muscles are centrally situated. EINARSON thinks that cells innervating "anti-gravity muscles" possibly possess a special power of resistance.

Experimental. Anoxaemia in the Lumbar Part of the Spinal Cord.

My experiments completely confirm the superior power of resistance of the peripheral cells as compared with the central. It is a practically constant observation in almost all animals experimented upon that the cells in the center of the anterior horn degenerate much more rapidly than those in the circumference.

¹ Supported by a grant from The King Christian X Foundation.

The experiments are made as follows: Anoxaemia of the lower part of the spinal medulla in rabbits is produced by means of a Häggqvist clamp (described by REXED 1940). I have modified this clamp by arranging a rubber bulb in a linen bag on the arm which presses the aorta against the central face of the spinal column (fig. 1). The rubber bulb is connected with a manometer. When air is blown in, the bulb can expand only to a certain point determined by the size of the bag before the pressure begins to rise. In the original Häggqvist model the arm pressing against the aorta is provided only with a metal plate and I could not always be certain to obtain occlusion of the vessel.

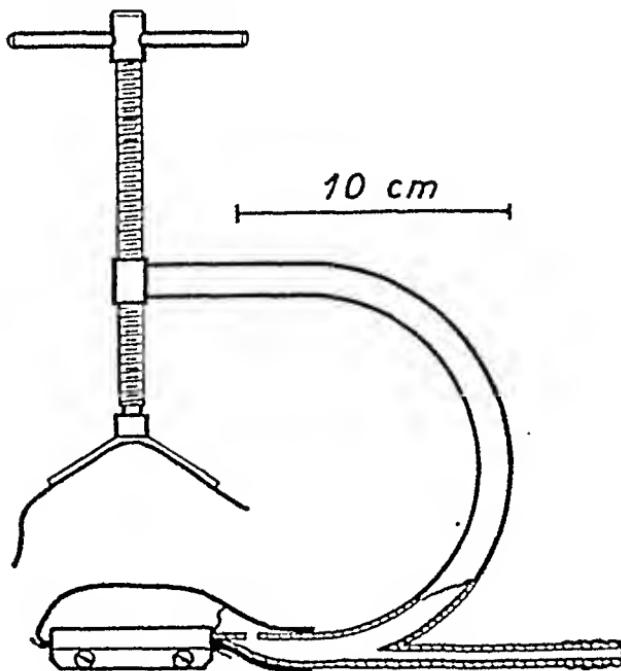


Fig. 1.

The following procedure is adopted: The rabbit is narcotized with ether and stretched on a board, belly downwards. The clamp is placed immediately behind the 12th rib, the arm with the rubber bulb against the ventral face of the spinal column. The clamp is slowly screwed down, the visera being moved away with a finger. Towards the close the aorta is palpated distally to the clamp and when the pulse is definitely weakened the rubber bulb is blown up to a pressure of 300 mm Hg which finally abolishes the pulse.

Before the introduction of the rubber bulb the occlusion was less certain and lesions of the abdominal organs were occasionally observed.

In these experiments it was a constant find that after an occlusion of 10—30 minutes the nerve cells in the central part of the anterior horn were definitely more damaged than those in the circumference (fig. 2). The figure shows the left anterior

horn from the sixth lumbar segment. A number of well preserved nerve cells are seen in the circumference while all nerve cells have disappeared from the center. The cells in the right hand lower corner are swollen and are no doubt in a state of pathologic activity as described by the author in a paper (E. KROGH 1945). Compare fig. 3 showing the same segment from a normal rabbit.

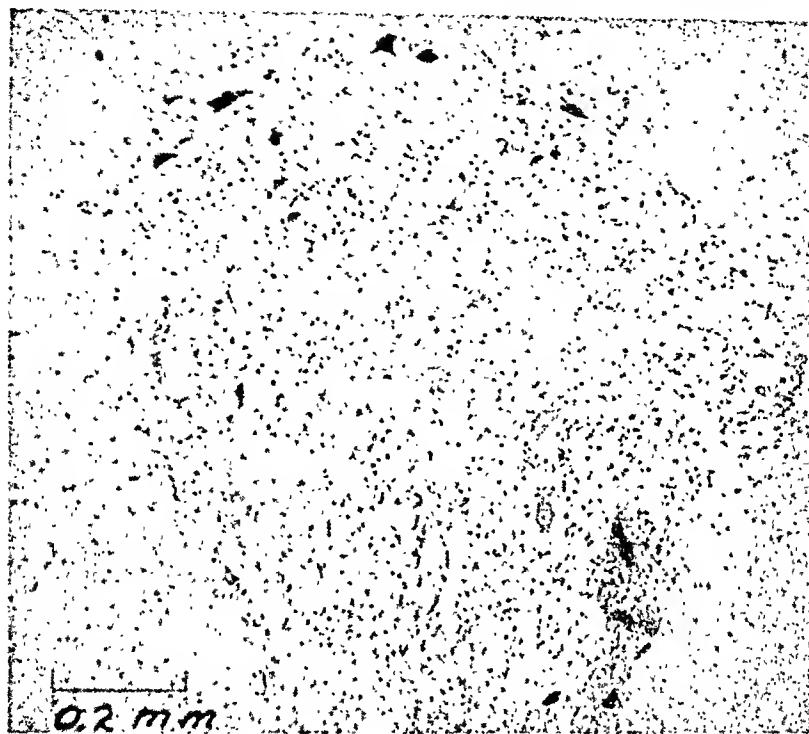


Fig. 2. Left anterior horn, lumbar segment 6. Aorta occluded 20 min., animal killed 48 hours later; well-preserved nervecells at the periphery of anterior horn; nervecells in pathological activity in right lower horn.

Fig. 4 shows the anterior horn from the sixth lumbar segment occluded 1 hour and killed 48 hours later. All nerve cells have disappeared with the exception of some degenerated remnants above and to the left. The glia cells however have proliferated near the edge of the horn while the center shows degeneration also of the glia and all other tissue elements. Not only the nerve cells, but also the glia cells show greater resistance against anoxaemia in the circumference of the anterior horn than in the center.

These pictures do not represent unique finds from my material of 50 rabbits, but are typical for all cases in which the duration of occlusion and time of survival were suitable.

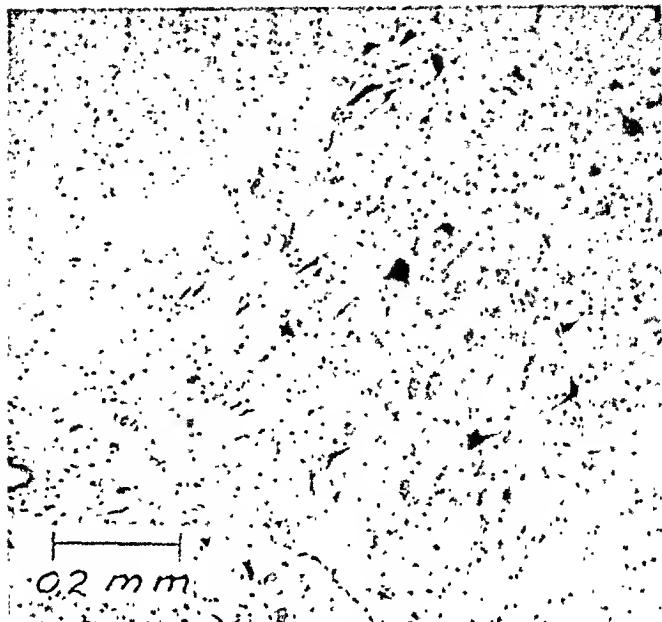


Fig. 3. Normal right anterior horn, lumbar segment 6.

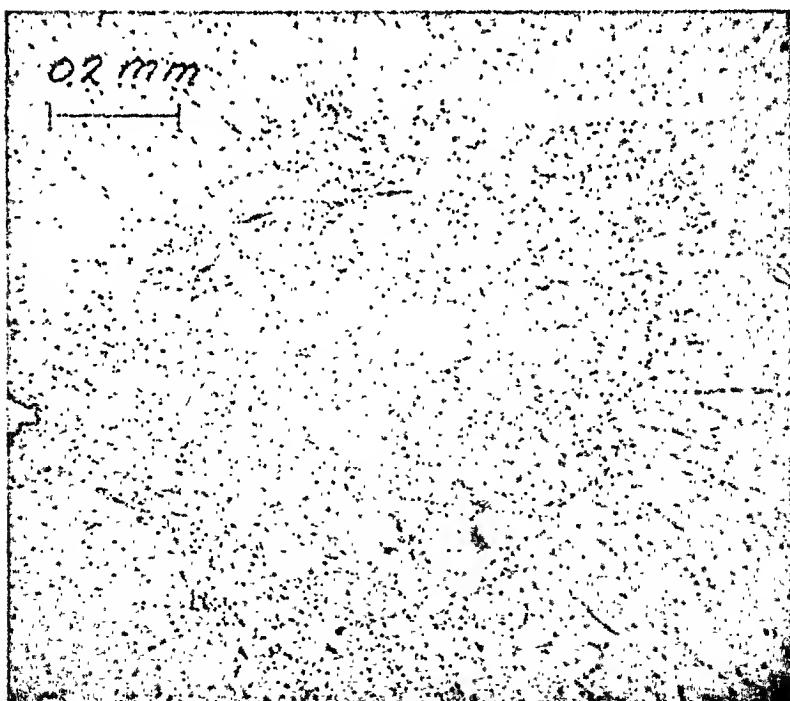


Fig. 4. Right anterior horn, lumbar segment 6. Aorta occluded 1 hour, animal killed 48 hours later. Nervecells disappeared, a few severely changed remnants left; proliferation of glia at the periphery of the anterior horn and degeneration of glia and other tissue elements in the center.

I am of opinion therefore that it is safe to conclude that the different power of resistance is due to some difference in the blood supply since there is no reason to believe that the glia (astrocytes, oligodendro- and microglia) in the periphery should have as *cells* a higher power of resistance than the quite similar cells in the center.



Fig. 5. Rabbit with paralysis of adductor muscles.

As regards which muscles are first paralysed it was interesting to observe that in many rabbits the adductors and flexors were the least resistant. Fig. 5 shows a rabbit with paralysis of the adductor muscles.

Fig. 6 shows a rabbit with rigidity of the extensors and the muscles on the dorsal face of the crus (the anti-gravity muscles). Flexors and adductors in this rabbit were completely paralysed. The histological picture of the anterior horn in this rabbit was just as presented above: Central nerve cells degenerated, peripheral preserved. The peripheral cells exhibited the condition of the pathological activity also shown in fig. 2.

To make out the exact localization of the fields of innervation for the different groups of muscles it will be necessary to do experiments according to the principles of chromatolysis (transsection of axons) and this will be the prerequisite to settle the theory concerning a special power of resistance (due to the blood supply) in the anti-gravity muscles.



Fig. 6. Rabbit with rigidity of antigravity muscles.

An Investigation of the Vascularization.

In order to study the special conditions of the blood supply I first attempted like TUREEN (1936) to inject 2 cm³ of a 4 % solution of neutral red into the ear vein of a rabbit having the abdominal aorta occluded as in the experiments. The animals were killed after 30 min., the medulla exposed and illuminated with ultraviolet light. This causes the tissue containing neutral red to light up. The experiments did not show the presence of any neutral red below the 3rd lumbar segment. I have also tried to examine frozen sections from the medulla by means of the luminescence-microscope but found no trace of neutral red below the 3rd lumbar segment.

Next I injected colloidal graphite (after DRINKER and CHURCHILL 1927) into the ear vein of a rabbit having the aorta occluded as usual. The circulation remains normal for 5 min. only, when the graphite agglutinates and the animal dies. In this case single grains of graphite could be found even in the sacral medulla, showing that some slight circulation must be maintained during the occlusion. This graphite experiment is too unphysiological and it became desirable to find a harmless substance which could be easily recognized. Such substances are now available in the isotopes of common elements. Before I could obtain these I tried the dye T 1824 which is related to trypan blue. It is non toxic and is used for determination of blood volume in men. It stays in the circulation for quite a long time. It turned out to be impossible to fix the dye in the tissues and only a very rough estimate of the degree of anoxaemia could be obtained. The next step was to utilize radio-sodium (²²Na). Such strong preparations could be obtained by courtesy of the Institute for Theoretical Physics (Prof. N. BOHR) that the substance could be recognized in a dilution of 1 to 450 millions.

The experiments are made as follows: The animal is narcotized, the aorta occluded, the radio Na injected into an ear vein as chloride in isotonic solution colored with T 1824. After a period, varied in the different experiments between 5 and 30 min., the animal is killed by decapitation, the thorax opened and only when the heart has stopped beating is the clamp removed.

The medulla is removed. By means of the T 1824 coloration the ischaemic zone is roughly ascertained and the medulla is cut up into 3 pieces, one with normal circulation, one representing the transition zone and finally the ischaemic zone proper. These pieces are placed on 3 separate freezing tables in a Petri dish and frozen by means of dry ice in a Dewar vessel. The freezing table is a circular brass plate of 9 cm diameter on to which is soldered near the edge a piece of brass tubing 35 mm in diameter and 13 mm high.

When a piece is frozen the freezing table is placed on the desk. It is now possible to regulate the temperature as required by means of a mixture of alcohol and dry ice in the short piece of tubing. The pieces of medulla are kept just lightly frozen. Slices of about 2 mm are cut, and from these samples are pricked out by means of a cannula ground

to a fine edge as a cork borer. The largest cannula employed had an internal diameter of 0.6 mm. By means of these cannulas it is possible to secure samples exactly localized in the grey or white matter.

The samples are distributed into the special aluminium dishes used in the Geiger-Müller counter. The sample is emulsified with a little distilled water, dried in an oven and weighed to 0.01 mg when it forms a very thin film covering the bottom of the dish. It is important to have nearly the same amount of tissue in each dish as the radiation is to some extent absorbed by passage through the film. The activity of each sample is finally determined and corrected as described (LEVI 1941, HOLM-JENSEN 1943) so that the different preparations can be directly compared.

An experiment without occlusion showed the relation between the activities of the grey matter in the anterior horns

$$\frac{\text{activity in lumbar part}}{\text{activity in cervical part}} = \frac{105}{100}.$$

What is measured is really the relative quantity of blood in the grey substance. Professors KROGH and HEVESY informed me that the blood vessels in the central nervous system are so slightly permeable that the Na ions need 60 hours to come into equilibrium with the water phase outside the vessels. These vessels are highly permeable only for water and fat soluble substances including oxygen.

The accuracy of the determinations does not exceed 5 per cent, so that the above result cannot indicate any difference in vascularization between the cervical and lumbar medulla.

In the following the activity obtained from the cervical anterior horn, where the circulation is normal, is put at 100.

In the second experiment in which the abdominal aorta was occluded as usual and the animal killed 5 min. after the injection of ^{22}Na relative activity in the lumbar anterior horn was found = 7.2.

In a third quite similar experiment the activity about the middle of the lumbar medulla was 16 and in the lower lumbar + sacral medulla 7.2.

In a 4th experiment in which the animal lived under occlusion for 30 min. after the injection I found the activity in the upper lumbar medulla = 53 and in the lower 15.

A similar experiment (No. 5) was made on a dog. In this case the occlusion was performed after TUREEN (1936). Under narcosis and artificial respiration the thorax was opened on the left

side and the aorta clamped directly at the 3rd thoracal segment. ^{24}Na was injected and the animal killed after 15 min. The relative activity in the lumbar medulla was 35.

From these experiments it can be deduced: In the brief experimental period of 5 min. practically no exchange of Na ions takes place between the blood and the extracellular fluid in the medulla. What we measure is therefore the admixture of blood from above the occlusion to that below. When the blood is completely mixed the activities in the lumbar and cervical parts must become identical, when the blood volumes present are the same.

Exp. 1 without occlusion shows this to be the case. *Exp. 2* and *3* show that only about 7 % oxygenated blood reached the lower part of the lumbar medulla but 16 % the middle part in 5 minutes.

In *exp. 4* of 30 min. duration a definite admixture of oxygenated blood has taken place viz. 15 % in the lower lumbar medulla and about 50 % in the middle and the experiment with the dog shows admixture of 35 % after 15 minutes in spite of the high occlusion.

The obvious conclusion is that an extremely slow circulation takes place in the occluded part requiring an hour or more for complete exchange of the blood in the capillaries. Normally this exchange takes place in much less than 1 minute.

The Course of the Vessels in the Anterior Horn of the Medulla.

I have tried to elucidate the problems by means of injection preparations. The injections were made in the main on rabbits, but also on a monkey (*Macacus rhesus*) with India ink-gelatine as follows: The main carotid is exposed on one side and the jugular vein on the opposite. A glass cannula is introduced in the carotid directed towards the heart and the peripheric part ligated. The exposed jugular vein is opened. The system is first washed out with warm Ringer saturated with oxygen until the fluid runs out unstained with blood from the jugular and then the gelatine is injected and the injection continued until the stained gelatine comes out only slightly diluted from the internal jugular. In this way it is attained that the veins are most completely filled with India ink close to the capillaries and less in their central parts which makes it easier to distinguish between veins and arteries in a preparation cleared to transparency; as it is necessary only to observe a vessel over a certain distance to make out its character of artery or vein.

After the injection the medulla is removed and fixed and slices of about 1 mm thickness are cleared according to AURELL (1942). The principle of this method is to determine the refraction index by clearing the fixed tissue, after careful dehydrating, in solutions of methyl salicylate and benzyl benzoate in varied proportions and corresponding varied refraction. The tissue shows the same refraction as the solution giving the most perfect clearing. By these experiments it was found that the medullar tissue was best cleared in a solution with a refraction index of 1.552. The myelin, however, could not be cleared by the method of AURELL — as he mentions himself in his paper — but I succeeded in dissolving out the myelin completely from my preparations by fixing in Carnoy's solution (1 part glacial acetic acid, 3 parts chloroform and 6 parts alcohol) and later treating the pieces with absolute alcohol, dioxane and chloroform.

Applying this clearing technique it became possible to follow the course of the blood vessels in slices of the medulla of 1 mm thickness. It turned out that there was no difference in the density of the capillary network in the central and peripheral parts of the anterior horn respectively. The high power of resistance of the cells in the periphery does not therefore depend upon a more abundant vascularization.

It was found on the other hand that the arteries, even those passing through the center of the anterior horn, split up into capillaries mainly near the circumference while the veins are formed mainly at the center. The cells in the periphery are therefore on the whole situated near the arterial ends of capillaries and the central cells near the venous ends.

It is difficult to demonstrate this by mierophotography on account of the small depth of focus of the stereo microscope at the magnification necessary. Only by direct observation and continuous change of focus does it become possible to follow the course of the vessels in the thick slices.

After some practice it becomes easy to distinguish between arteries and veins in the cleared preparations as mentioned by PFEIFER (1928) and by CAMPBELL (1938). According to CAMPBELL the arteries are characterized by a straight course, their walls are smooth, the lumen definitely circular and they give off branches chiefly by dichotomic ramification with branches of nearly the same calibre. The veins show a more irregular course and cross section and are made up by affluents of irregular distribution and greatly varied thickness.

My studies fully confirm these statements and by the injection technique employed by me it becomes even easier to distin-

guish between arteries and veins when the vessels are followed for some distance.

The result of the examination of cleared preparations is therefore that the cells in the periphery of the anterior horn are in the main close to the arterial ends of the capillaries, while the central cells lie close to the venous ends.

By the very slow circulation produced by the occlusion the peripheral cells will have an opportunity to use up most or all of the oxygen available, and this is the reason why they better resist the lack of oxygen.

It is probable that for the same reason these same cells better resist a lack of E-vitamin than the central ones (EINARSON and RINGSTED). Differences in concentration of the vitaminin between the arterial and venous ends of capillaries are possible even with the normal rapid circulation, because the E vitamin, like other fat soluble substanees, diffuses very rapidly through the capillary walls in the central nervous system. (A. KROGH 1945 in press.) The same argument will hold also for the A vitamin and probably explain the observation referred to by E. MELLANBY.

Summary.

The paper deals with the special vascularization studied in the anterior horn of the medulla, mainly in rabbits.

In the first part it is shown that the cells in the periphery of the anterior horns are more resistant towards laek of oxygen produced by occlusion of the abdominal aorta than the eentral cells. Since this is true both for nerve cells and, after prolonged ocelusion, also for glia cells the difference must be due to a difference in the blood supply.

In the seeond part of the paper it is shown by means of radio sodium that a very slow circulation takes place in the anoxaemic part of the medulla during oeclusion. The exehange of blood in the capillaries normally requiring less than a minute now takes an hour or more.

In the third part of the paper it is shown by means of injection preparations that there is no difference in the density of the capillary network between the center and the periphery, but that the arteries split up into capillaries mainly at the periphery while the veins are formed mainly near the center.

The tissue best resisting the occlusion lies therefore close to the arterial ends of capillaries and the least resistant close to the venous ends, and with the extremely slow flow during occlusion the peripheral cells can appropriate most or all of the available oxygen.

The placement of a cell relative to the course of a capillary from the artery to the vein is essential for its oxygen supply when the circulation is much reduced by occlusion.

The experiments of EINARSON and RINGSTED on long standing lack of E vitamin in rats point to the conclusion that this relation may also be significant for rapidly diffusing substances of very low concentration at normal rates of circulation, since it is shown that the peripheral cells in the anterior horns of the medulla innervating extensors and supinators (anti-gravity muscles) better resist an almost complete lack of E vitamin than the cells in the central part (adductor and flexor muscles).

Literature.

AURELL, G., Z. wiss. Mikr. 1938. 55. 256.
 AURELL, G., Ibidem, 1942. 58. 113.
 BOK, S. T., Hdb. mikr. Anat. Menschen, 1928. 4. 478.
 CAMPBELL, A. C. P., Res. Nerv. Ment. Dis. Proc. 1938, 18. 69.
 DRINKER, C. K., and E. D. CHURCHILL, Proc. Roy. Soc. 1927. 101B. 462.
 EINARSON, L., and A. RINGSTED, Effect of Chronic Vitamin E Deficiency on the Nervous System and the Skeletal Musculature in Adult Rats, Copenhagen and London, 1938.
 HOLM-JENSEN, I., Acta physiol. Scand. 1943. 5. 271.
 KROGH, A., Proc. Roy. Soc. (in press).
 KROGH, E., Acta Jutlandica 1945. Supplementum XVII.
 LEVI, HILDE, Acta physiol. Scand. 1941. 2. 311.
 MELLANBY, E., The Advance of Medical Science since Huxley's day. The Huxley Lecture, London 1934.
 REXED, B., Acta Psych. Neur. 1940. 15. 365.
 PFEIFER, R. A., Quoted by Campbell.
 TUREEN, L. L., Arch. Neurol. & Psychiat. 1936. 35. 789.
 TUREEN, L. L., Res. Nerv. Ment. Dis. Proc. 1938. 18. 394.

From the Chemical Department of Karolinska Institutet and
the State Bacteriological Laboratory, Stockholm.

Studies on Nucleic Acid Metabolism in Plasma Cells.

By

JENS BING, ASTRID FAGRÆUS and BO THORELL.

Received 12 August 1945.

Introduction.

I. In the course of the last few years increasing interest has been taken in the function of the plasma cells, as it has been shown to be probable that those cells play some part in the formation of pathological serum-globulin, Bence-Jones' albumose, amyloid and presumably also — at any rate in part —, the normal serum-globuline.

Proceeding from myeloma studies, MAGNUS LEVY 1932 and HECHT JOHANSEN 1934 considered that the formation of globulin proceeded from the bone-marrow cells, but they failed to find any special type of cell that could be regarded as a globulin producer. In 1937, however, BING and PLUM showed that a feature common to those diseases which entail a marked hyperglobulinemia was an increase in the plasma cells and other reticulo-endothelial cells within and outside the bone-marrow, which is an indication that the formation of globulin proceeds precisely in those cells. This supposition has since been confirmed by a number of other authors on the basis of larger material. It was shown that the same relation between hyperglobulinemia and plasma cells as well as other reticulo-endothelial cells was found in animals (pigs, horses, cows, rabbits) with a spontaneous or experimentally induced hyperglobulinemia (BING and CHRISTENSEN 1944). It has likewise been shown that an isolated increase of globulin in the spinal fluid (BING and NEEL 1942) occurs only in those diseases where there is an accumulation of plasma

cells in the central nervous system and its membranes, and that in nasal polyps (ANDERSEN and BING 1944), rich in plasma cells, more globulin may be found than in the same patient's serum. Finally, it may be mentioned that, after intracutaneous injection of antigen at the time of the local antibody formation, an accumulation of plasma cells and other reticulo-endothelial elements in the skin can be observed. The relation between antibody globulin and plasma cells has been specially studied by BJÖRNBOE and GORMSEN 1941, who have shown that in rabbits treated with polyvalent pneumocoecal vaccine — in addition to hyper-globulinemia — a marked increase in the number of plasma cells also occurs.

II. On the basis of a large material, CASPERSSON and coworkers have shown that, in the Metazoa cell, a new formation of protein in general proceeds in the presence of nucleic acids. Broadly speaking, we can distinguish two protein-formation processes, one for the new formation of gene protein during mitosis, and one for the new formation of cytoplasmic protein. The new formation of gene protein is effected by ribodesose nucleotides, which during the prophase accumulate on the chromosome elements, whereas the new formation of cytoplasmic protein is promoted by ribose nucleotides. The mechanism that regulates the new formation of cytoplasmic protein includes certain cell organelles, such as the chromocentre, the nucleolar apparatus and the nuclear membrane. From the stage of development of these organelles, especially the amount and composition of the nucleolar material and the cytoplasmic content of ribose polynucleotides formed from the nuclear membrane, the functional state of the cytoplasmic-protein-formation system in individual cells can be determined. This has been shown to apply also to cells where the newly formed protein will not be utilized by the cell body itself, but be given off to the environment, as, occurs, for example, in serous glands in their production of protein-containing secretions.

A study of the cells in the blood-forming organs in man and certain mammals (THORELL 1944, 1945) has shown that also these special types of cells, during their new formation of proteins in the growth phase, have a similar cytochemical organization as that of cell types previously investigated by CASPERSSON and coworkers, for example embryonal cells (CASPERSSON and THORELL 1941). Thus, on analysis with spectrographic and micro-chemical methods, a comparatively large nucleolar mass, containing ribose

polynucleotides, could be shown in the hemocytoblast, and in the cytoplasm high concentrations of ribose nucleotides. During the differentiation towards maturer forms, the concentration of nucleotides in these cells falls to nil, parallel with a gradual cessation of the growth of the individual cell (for details, see THORELL 1944).

A closer study of the cytology of the nucleolus indicated that the nucleolar mass during the interphase is formed by a special part of the nucleus, containing ribodesose nucleotides and termed the nucleolus-associated chromatin. The quantitative changes proceeding, during maturation, in the protein-forming mechanism were attributed to a diminution of activity in the nucleolus-associated chromatin. The formation of ribose polynucleotides seems to proceed in the centre of the nucleolus-associated chromatin, whence, according as a nucleolus containing ribose nucleotides is developed, the nucleolus-associated chromatin will form a thin outer layer surrounding the nucleolus. (The stage of development of the nucleolus can thus be determined from the picture of the nucleolus-associated chromatin, as shown on a preparation stained in accordance with the Feulgen nucleal test.) During the maturation of the blood cell, the activity of the nucleolus-associated chromatin diminishes, so that in the mature nucleus-containing cell no nucleolus rich in ribose polynucleotides develops: instead, the nucleolus-associated chromatin can be distinguished in the nucleus as a chromocentre, consisting solely of ribodesose polynucleotides.

With similar cytochemical methods as those mentioned above, myeloma cells were examined by THORELL and WISING 1944, in order to ascertain whether the hyperglobulinemia frequently occurring in cases of myeloma might be due to a production of protein from the myeloma cell itself. They found that the myeloma cell in general possesses the characteristics of a cell the function of which subserves an intense new formation of proteins, *i. e.* it has a large nucleolus, containing ribose polynucleotides. The myeloma cell was thus found to be of a similar cytochemical type as the growing blood stem-cell or the protein-secreting gland cell.

The above-mentioned studies afford facilities for cytochemically tackling the question as to the possible production of protein by the plasma cells. The primary object of the present investigation was thus to endeavour to ascertain whether plasma

cells in general possess the cytochemical organization which, according to CASPERSSON and coworkers, promotes a production of protein cytoplasmic protein during the growth of the cell or as a production of protein-containing secretion.

In the present study, living plasma cells from rabbits with experimentally induced hyperglobulinemia were examined with ultraviolet microscopy, in order roughly to determine the distribution of nucleotides in the cell; with the Feulgen nucleal reaction for the qualitative distinction between ribose and ribodesose nucleotides; and by staining with acid and basic dyes, the latter with special regard to the development of the nucleolus.

Material.

An increase in the plasma cells and hyperglobulinemia was induced in rabbits in the following way.

a) Rabbits, weighing about 2 kg, were immunized with polyvalent pneumococcal vaccine. The method elaborated by BJÖRNEBOE and GORMSEN 1941 was adopted: after a few minor initial doses, 4 ml of a vaccine with a density of 10^9 bacteria per ml were given three times a week. After 2—3 months the serum proteins and the pneumococcal antibody titre were determined, whereupon biopsy of the spleen was performed. For the values, see Table I.

Table I.
Rabbits immunized with pneumococcal-vaccine.

Rabbit No:	Titer against pneumococcal- types							Serum proteins			
	1	2	4	7	8	12	14	Total protein	Globu- lin	Albu- min	rel. Alb %
1.....	640	160	320	640	640	40	0	6.74	3.02	3.72	55
2.....	320	160	640	640	640	160	40	7.37	3.60	3.77	51
3.....	640	320	320	640	1,280	320	80	7.16	3.44	3.72	52
4.....	640	320	320	320	1,280	320	80	8.13	4.05	4.08	50
5.....	320	320	320	320	640	80	0	6.80	3.11	3.69	54
6.....	1,280	320	320	320	640	160	0	7.47	3.13	4.34	58
7.....	1,280	160	640	320	320	320	0	7.57	3.39	4.18	55
9.....	1,280	160	640	640	640	320	0	7.53	4.33	3.20	43
10.....	320	640	640	320	1,280	80	160	7.13	3.72	3.41	48
11.....	1,280	320	640	640	640	320	0	7.38	4.27	3.11	42
12.....	2 560	320	640	640	1,280	80	40	7.38	4.56	2.82	38

b) In other cases, plasma cells and hyperglobulinemia were studied in serum disease in rabbits, induced by the injection of horse serum (the method indicated by FLEISCHER and JONES 1932). First, a massive dose (20—30 ml) was given, and afterwards smaller doses (10—15 ml) in the course of two succeeding days. See Table 2.

Table 2.

Rabbits immunized with horse serum.

Rabbit N:o	Weight	Injected amount horse serum	Days after 1:st inj.	Titer (antigen dil. method, serum diluted 1:2)
269	2,300	20 ml × 1:st day	7	1/800
283	2,200	16 ml × 1:st day	8	1/500
273	2,400	30 ml × 1:st day	9	1/3,200
		15 ml × 2:nd day		
274	2,100	as 273	11	1/6,400
275	2,600	as 273	15	1/12,800

With a view to obtaining differences between normally functioning plasma cells as compared with cells which might be presumed to be engaged in an intense new formation of antibodies, rabbits were syringed with a certain dose of horse serum, whereupon they were killed at intervals of a few days and the plasma cells were examined (mainly from the milt). The first rabbits thus represented the incubation stage, the following ones the phase of rapid increase in antibodies, and the last ones the constant titre level. (Table 2.)

The nucleotide distribution in the plasma cell.

In order roughly to examine the distribution of nucleotides in the individual plasma cell, microphotographs were taken of living plasma cells immersed in a physiological saline solution, in monochromatic ultraviolet light of the wave-length 2570 Å, thus very near the absorption maximum of the nucleic acids (see CASPERSSON 1936). As the nucleotides, relatively to other substances occurring in biological material, dominate the light absorption at this wave-length, the parts especially rich in these substances appear brought out on a microphotograph as dark areas (see *loc.cit.*).

For a qualitative distinction between ribodesose and ribose nucleotides in the cell, the Feulgen nucleal reaction was adopted. It was performed on fixed smear preparations of plasma cells. After acid hydrolysis and lipid extraction, the plasma cells were treated with para-fuchsine sulphuric acid, which with the free ribodesose-aldehyde group assumes a red colour. As other substances with a true aldehyde group after acid hydrolysis and lipid extraction are rarely found in biological material, the Feulgen reaction may be regarded as specific for ribodesose polynucleotides (see FEULGEN and ROSENBECK 1924).

On investigation with the above-mentioned methods, the following results were obtained (fig. 1 and 2).

The nucleus of the plasma cells, besides minor, ultraviolet-absorbing chromatin grains, contained also a few large, intensely absorbing nuclear bodies.

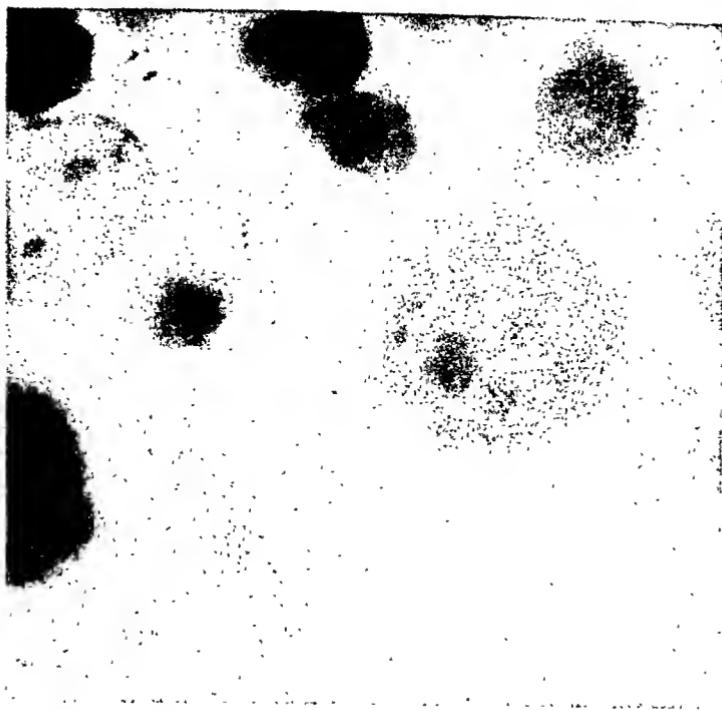


Fig. 1. Ultraviolet picture of an unmatured plasma cell (a-cell, see text). Wavelength 2,573 Å. Objective aperture 1.25. Condenser aperture 0.9. Magnification 4000 \times . Plate: Agfa Platten B. Development: alkaline hydroquinon 5 minutes.

The cytoplasm was found to contain an abundance of ultraviolet absorbing substances. Close to the nuclear membrane, especially at the part lying nearest the centre of the cell, there was a more or less marked crescent-shaped area, almost entirely free from light absorption. The other, larger part of the cytoplasm showed an intense absorption at 2570 Å, $\log I_0 - \log I_1$, photographically measured as 0.5 corresponding to a considerable nucleotide concentration (Table 3).

It was found possible, with a photographic technique described

¹ The curves were obtained in the following way: The cell object was illuminated with monochromatic ultraviolet light, coming from a spark. For details of arrangement, see KÖHLER 1932. The monochromatic radiation adopted was: 3100, 2930, 2800, 2750, 2570, 2510, 2440 and 2395 Ångström units. Diffuse light was filtered off by Cl₂ and a solution of NiSO₄ and CoSO₄. The object was photographed under strictly constant illumination conditions. Objective aperture 0.85 and condenser aperture 0.6. A rotating sector, divided into 16 steps was photographed at the same time. Time of development 3 minutes. A self-registering photometer was used in measuring the density. From the density values of the sector and the cellular detail, the light absorption of the cellular detail was determined graphically.

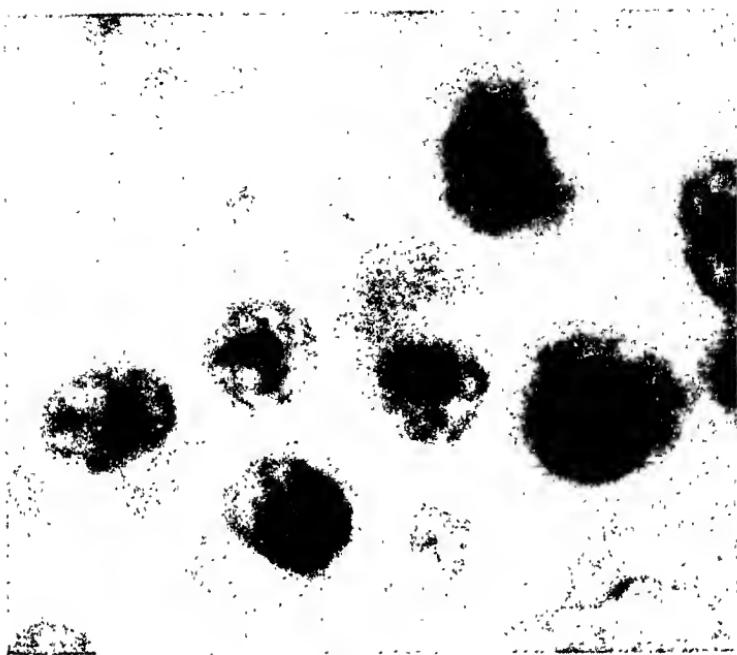


Fig. 2. Ultraviolet picture of mature plasma cells (b-cells) Magnification 4000 \times .
For other data, see Fig. 1.

by THORELL 1945¹, to take complete absorption spectra of the cytoplasm in living plasma cells, within the wave-length range in question, 3100 Å—2400 Å. After correction for light diffrac-

Table 3.

Extinction coefficients at 2570 Å of the cytoplasm in a-and b-types of plasma cells. Each value corresponds to the cytoplasmic absorption in a single cell. For the method used, see Thorell 1944.

Type a					Type b				
0.43	0.42	0.44	0.41	0.45	0.42	0.45	0.48	0.44	0.52
0.49	0.50	0.36	0.40	0.62	0.56	0.60	0.53	0.50	0.38
0.63	0.48	0.49	0.58	0.62	0.52	0.45	0.52	0.49	0.47
0.44	0.50	0.54	0.52	0.53	0.57	0.52	0.45	0.47	0.39
0.51	0.36	0.46	0.58	0.42	0.57	0.39	0.40	0.59	0.39
0.38	0.63	0.55	0.56	0.53	0.38	0.38	0.58	0.45	0.40
0.63	0.62	0.61	0.64	0.50	0.58	0.62	0.60	0.49	0.40
0.52	0.10	0.38	0.36	0.47	0.50	0.58	0.54	0.57	0.49
0.62	0.52	0.50	0.56	0.58	0.44	0.57	0.66	0.47	0.51
0.64	0.52	0.37	0.53	0.48	0.63	0.72	0.46	0.49	0.63
0.42	0.57				0.42	0.41	0.45	0.44	0.54
Mean value = 0.51 \pm 0.012					0.46	0.54	0.59	0.66	0.53
σ = 0.083					0.60	0.72	0.48	0.62	0.42
					0.16	0.41	0.39	0.36	
					Mean value = 0.50 \pm 0.010				
					σ = 0.087				

tion and reflexion, such a light absorption curve shows a maximum at 2600 Å (fig. 3), which under certain conditions can be looked upon as specific for the pyrimidin groups contained in the nucleic acids (see op. cit. 1936).

After the performance of the Feulgen reaction, only the "chromatin" assumed colour (fig. 4 and 5). The intensely absorbing cytoplasm at 2570 Å gave an entirely negative result, indicating that the light-absorbing substances consisted of ribosepoly-nucleotides.

The large, intensely absorbing nuclear bodies appeared on the Feulgen-stained preparation as Feulgen positive "ring formations", limiting an entirely colourless, central part of varying size. In the plasma cells, which, for several reasons (see below) were presumed to be young, a large Feulgen-negative nucleolus, surrounded by a regular, nucleal-positive outer layer, was observed. In many of the typically mature plasma cells, on the other hand, one could merely distinguish a central clearing in the otherwise completely Feulgen-positive nuclear body.

Summing up, we obtain the following picture of the essential cytochemical organization of the plasma cell in general:

The cytoplasma, except at a small area nearest the nuclear membrane, contains considerable concentrations of ribose poly-nucleotides.

In the nucleus there are one or more largish accumulations of chromatin containing ribodesose nucleotides, which, like the nucleolus-associated chromatin in young blood-cells in the earlier stages of growth, has developed a centrally situated nucleolar mass, containing ribose-polynucleotides. The amount of this nucleolar substance varies considerably, but we cannot, as we find in the mature leucocyte, observe plasma cells with a completely

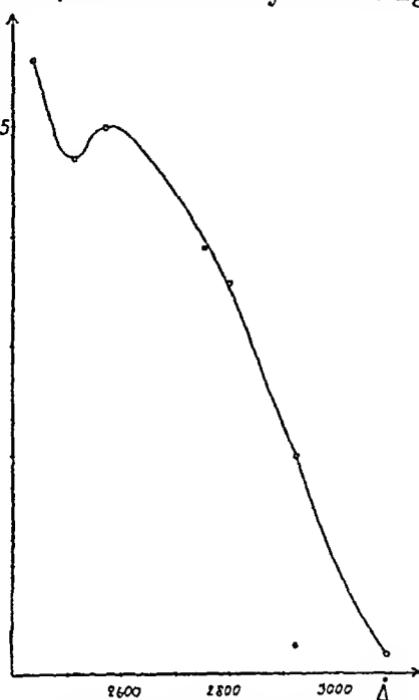


Fig. 3. Ultraviolet absorption spectra of the cytoplasm in a mature plasma cell. In the curve appears the nucleic-acid band at 2,600 Å. For measurement data, see text.

inactive heterochromatique area, containing ribodesose nucleotides, in the nucleus.

As mentioned above in the desription of the piture of the nucleolus-assoeiated ehromatin, a further result of the investigation was that a classification of plasma cells could be made on the basis of eertain quantitative cytochemical and cytological data. This was shown very distinctly during the successive examination of plasma cells after the injection of horse serum into rabbits.

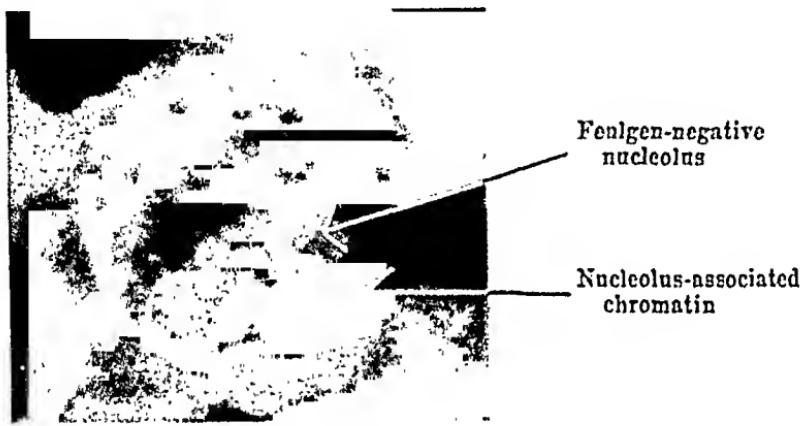


Fig. 4. Feulgen-stained a-cell. Observo the layer of disrupted nucleolus-associated chromatin.

The two extreme types of plasma cells may be described as follows:

a) (Fig. 1 and 4). Cells with a high nucleus-plasma relation and a well-developed nucleolar-mass, the nucleolus-associated ehromatin in the Feulgen-stained preparation being observable as a thin, nucleal-positive outer layer, surrounding the nucleolus.

b) (Fig. 2 and 5). Cells with a relatively low nucleus-plasma relation and an excentrically situated nucleus, containing ribodesose-nucleotide chromatin, caked together. The ribose-poly-nucleotide nucleoli were quite small, being observable on the Feulgen preparation merely as central clearings in the Feulgen-positive nucleolus-associated chromatin.

Estimated on the basis of the light absorption at 2570 Å, both types of cells, broadly speaking, had *the same high concentration of ribose polynucleotides in the cytoplasm*. (Table 3).

Cell type a) was most frequent during the ineubation stage (Table 4), that is, during the period when the most intensive

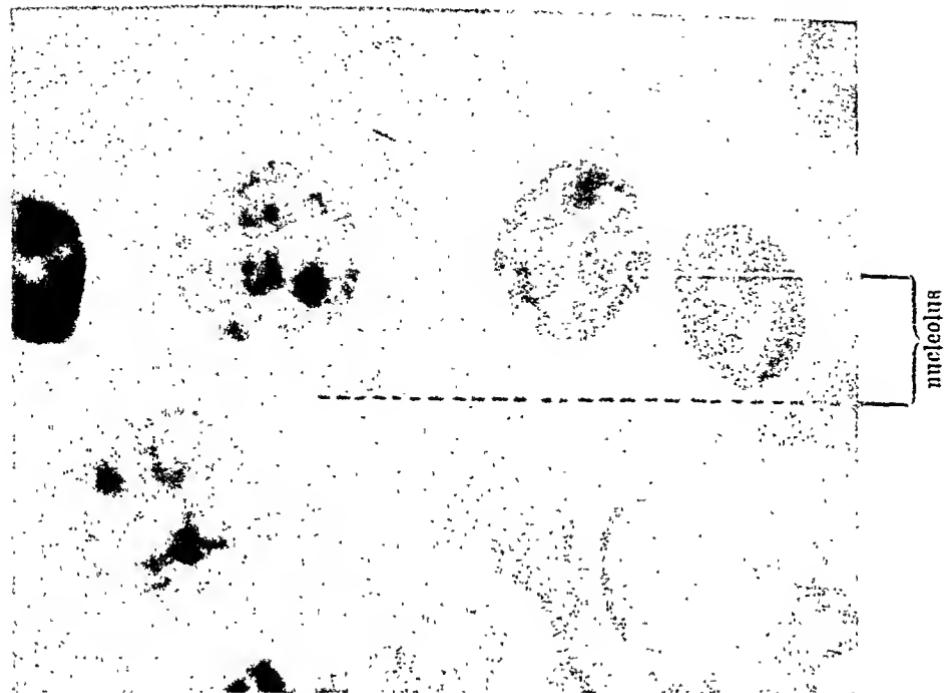


Fig. 5. Feulgen-stained b-cells. The Feulgen-negative nucleolus is very small and surrounded by a thick layer of nucleolus-associated chromatin.

new formation of plasma cells was taking place. Cell type b) predominated during the high antibody titre period (Table 4).

Cell type b) entirely corresponded to the normal type of mature plasma cell. No cytochemical or cytological difference between plasma cells from normal rabbits and plasma cells from rabbits with a high antibody titre could be observed. Only the number was larger in the latter case.

Table 4.

Frequency of a-and b-types of plasma cells during immunization.

Rabbit N:o.	Antigen	Dose g	Time after 1:st injection	Titre or amounts of globuline	Type a	Type b	Number of counted cells.
283...	horse serum	16.0 (30×1)	8 days	1/500	20.1	1.1	452
273...	" "	(15×2)	9 "	1/3,200	24.1	4.5	331
274...	" "	as 273	11 "	1/6,400	25.4	7.7	639
275...	" "	as 273	22 "	1/12,800	10.6	22.0	622
B. 9..	pneumococcal vaccin, 7 types		3 months	4.33 %	12.7	28.5	544
B. 11.	D:o		3 "	4.27 %	11.2	25.6	609
B. 12.	D:o		3 "	4.56 %	9.35	20.5	534

Concluding survey.

This study has firstly confirmed BJÖRNBOE's and GORMSEN's finding that, concomitantly with a formation of antibodies, after immunization with polyvalent pneumococcal vaccine, a marked increase in the number of plasma cells is obtained.

Secondly, an increase in plasma cells was observed after the injection of horse serum into rabbits.

Parallel with the antibody titre, an increase of the globulin fraction in the serum was found.

The main problem was to endeavour to ascertain whether there might be any direct connection between the plasma cells and the large amounts of globulin, or, in other words, whether the plasma cells act as producers of globulin.

The investigation was based on the observations hitherto made, chiefly by CASPERSSON and his associates, in regard to the endo-cellular nucleotide metabolism in the new formation of protein (see the Introduction, section II). Those observations have in fact afforded facilities for estimating the intensity of the protein formation in the individual cell by a qualitative and quantitative determination of the nucleotide distribution in that cell.

According to the results of the analysis, the cytochemical organization of the plasma in general indicated a production of protein in those cells. But, on the basis of the observations hitherto made, it is not possible to determine from the cytochemical picture of the individual plasma cell, whether this protein synthesis is intended for the building-up of protein in the cell itself, or for the production of protein-containing secretion for the benefit of the environment.

During the first stage of the immunization, a marked increase in the number of plasma cells was particularly noticeable. The most frequently occurring type of plasma cell (a) was found to correspond, in cytochemical organization, for example, to the rapidly growing blood stem-cell, that is, a high nucleus-plasma relation, a well-developed ribose-polynucleotide nucleolar mass, surrounded by a thin outer layer of ribose-polynucleotide containing nucleolus-associated chromatin and, in the cytoplasm, high concentrations of ribose polynucleotides. It may be presumed with a great degree of probability that the nucleolar-cytoplasmic nucleotide mechanism of this type of cell subserves a new formation of plasma cells, or, in short, that the cytochemical and cyto-

logical picture of this type of plasma cell is a manifestation of cellular proliferation.

During the latter stage of the immunization, with the constantly high titre level, a type of cell, cytologically and cytochemically identical with the normally occurring mature plasma cell (b) predominated. During the transition from the type of plasma cell, engaged in rapid growth and occurring during the incubation stage (type a), to the later, mature plasma cell (type b), it seems — to judge by the nucleolar apparatus — that a similar process as during the maturation of the blood-cell takes place, namely a gradual diminuation of the amount of developed ribose-nucleotide-containing nucleolar mass (though never to nil), but with the difference that *the ribose-polynucleotide concentration in the cytoplasm remains unchanged*.

The marked increase of plasma cells during the incubation period and the large number of plasma cells during the stage of the high titre level (for data, see above), in conjunction with the results of previous investigations, renders it probable that the antibody protein is produced by these cells. The high concentration of ribose polynucleotides in the *cytoplasm* of the mature plasma cells indicates, according to previous observations, that extensive protein metabolism processes are going on in it. From these data, taken together, it may be inferred that *the formation of antibody protein proceeds essentially in a similar way as the protein synthesis in cells in general, namely in the presence of nucleic acids*. In the same mature plasma cell, however, the *nucleus* and its parts, showed, according to the observations hitherto made, a relatively small activity. Investigations into passive homologous sensitization have indicated (JORDAN 1941) that the increase of antibodies is *an autocatalytic process, guided by the antibody molecule*, and analogous with the gene and virus effect. This may explain why the nucleus in the mature plasma cell does not — as in other cells with a similar function (embryonal cells, pancreas, etc.) appear to take more active part in the cytoplasmic new formation of protein.

Summary.

In order to study the endocellular formation of antibodies, plasma cells produced by injection of polyvalent pneumococcal vaccine or horse serum in rabbits, have been analysed with

mierspectrographical and microchemical methods. The successive examination of plasma cells after the injection showed that during the first stage of immunization the most frequently occurring type of plasma cell in cytochemical organization corresponds to the rapidly growing blood stemcell. During the latter stage of the immunization, with the constantly high titre level, a type of cell, identical with the mature plasma cell predominated. The transition from the rapidly growing cell to the mature cell is similar to the maturation of the blood-cell, but with the difference that the ribose-polynucleotide concentration in the cytoplasm remains unchanged. Together with the immunological data, this seems to indicate, that the formation of antibody protein proceeds essentially in a similar way as the protein synthesis in cells in general, namely in the presence of nucleic acids.

Literature.

ANDERSEN, M. C. and J. BING, Acta path. microbiol. scand. 1944. 21. 455.
BING J., and N. O. CHRISTENSEN, Medl. bl. f. Den danske Dyrelægeforen. 1944. Acta med. scand. 1944. 116. 382.
BING, J., and A. V. NEEL, Acta med. scand. 1942. 111, 57.
BING, J., and P. PLUM, Ibidem. 1937, 92. 415.
BJØRNBOE, M., and H. GORMSEN, Nord. Med. 1941. 891.
CASPERSSON, T., Skand. Arch. Physiol. 1936, 73. Suppl. S.
CASPERSSON, T., Naturwiss. 1941. 29. 33.
CASPERSSON T., H. LANDSTRÖM-HYDÉN and. L. AQUILONIUS, Chronosoma 1941. 2. 111.
CASPERSSON T., and J. SCHULTZ, Nature 1939. 602.
CASPERSSON T. and B. THORELL. Chromosoma 1941. 2. 132.
FEULGEN R., and H. ROSENBECK, Hoppe-Seyl. Z. 1924. 135. 203.
FLEISCHER J., and P. JONES, J. exp. Med. 1932. 79.
JOHANSEN A. and A. HECHT, Acta med. scand. 1934. 82. 276.
JORDAN, P., Naturwiss. 1941. 29. 89.
MAGNUS LEVY A., Acta med. scand. 1938. 95. 217.
THORELL, B., Ibidem 1944. 117. 334.
THORELL B., 1945 (to be published).
THORELL B., and P. WISING, Nord. Med. 1944. 24. 1842.

From the Departments of Biochemistry, Anatomy and Medicine,
University of Rochester School of Medicine and Dentistry,
Rochester, N. Y.

Studies on Dietary Gizzard Ulcers in Chicks.

By

HENRIK DAM and HARRY L. SEGAL.

Received 28 August 1945.

During the last decade several investigators have studied a dietary condition in chicks in which the muscular stomach is ulcerated ("eroded"). DAM (1934) and DAM and SCHOENHEYDER (1934) found this condition present in chicks reared on an artificial vitamin K free diet and gave a preliminary description of it. The tendency to ulcers was then believed to be related to the vitamin K deficiency. It is now certain that this is not the case; the basal diets used in the present studies contain an ample amount of vitamin K substitute and yet produce ulcers in a very high degree. ALMQUIST and STOKSTAD (1935—1937) reported that the factor which protects against gizzard ulcers is present in alfalfa together with vitamin K and is fat soluble. ALMQUIST further elaborated on the subject in 1937 and in 1938 found that bile acids protected the chick gizzard from ulcer formation. Other substances such as milk (ALMQUIST, MECCHI and KRATZER, 1941), cartilage, and chondroitin (BIRD and OLESON, 1938) have also been reported to counteract gizzard ulcers. One of the latest contributors to the field is CHENEY (1938, 1940, 1942) who reported that cinchophen (phenyl-cinchoninic acid) "atophan" produced gizzard ulcers when fed to chicks receiving a normal diet. The fact that cinchophen can produce ulcers in dogs and cats had already been reported (WAGONER and CHURCHILL 1932, STALKER, BOLLMAN and MANN 1937, SCHWARTZ and SIMONDS 1935). CHENEY suggested the name Vitamin U for the anti-ulcer factor and stated that it is present in the "sterol fraction of

fat which is saponifiable" and is readily destroyed by heat. The designation "factor U" has already been proposed by STOKSTAD and MANNING (1938) for another factor essential in the nutrition of chicks. CHENEY further reported that the tendency to ulcers is due not only to lowered resistance of the tissue involved but also to gastric hyperacidity.

A more detailed account of the literature on this problem is included in the publications already quoted and in papers by LANSING, MILLER and TITYS (1939) and ESSELEN (1939).

In the past few years one of the authors (DAM) has investigated the gizzard-ulcer problem, the main results of which are here-with presented as well as the results of the more recent work jointly studied.

Choice of ulcer-producing diets for chicks: In experiments on vitamin E (DAM 1944) it was noted that the vitamin E free diet no. 182, the composition of which is given in table 1, gave rise to perforating ulcers in many of the animals which had survived the vitamin E deficiency for a sufficient length of time. It was observed that vitamin E in the form of 10 mg% d,L-alpha-tocopherol acetate failed to prevent the ulceration, but, as mentioned later in the present paper, its incidence and severity were more prominent in the absence of vitamin E. However, vitamin E deficient basal diets could not be used advantageously in the ulcer study because of the complications which arise therefrom, especially when fats are tested.

Addition of $\frac{1}{2}$ to 1 % of cinchophen to the artificial diet containing vitamin E hastened the onset of gizzard-ulcers. It was therefore decided to carry out most of the tests with artificial cinchophen-containing diets supplemented with vitamin E. In some of these diets the casein content was reduced from 15 % to 10 %. Such a reduction caused the animals to eat less with the result that a smaller amount of material was required for the test. The addition of cinchophen to normal commercial diets as proposed by CHENEY, has been tried, but since the results obtained were less pronounced than those produced by the artificial diets, this procedure was abandoned.

The effect of cinchophen is not confined to the gizzard, as it produces a general intoxication which diminishes growth and which in man is known to affect the function of the liver (RABINOWITZ 1930). The feeding of cinchophen diets also leads to neuromuscular disturbances early in the experiment.

Table 1.

Basal diet no:	182	203	293	340
Casein, alcohol-extracted ¹	15 g	10 g	10 g	10 g
Dried yeast, ether-extracted ²	10 g			
Dried yeast, not extracted ²		10 g	10 g	10 g
Corn starch		61.3 g	60.8 g	60.8 g
Sucrose	54.6 g			
Gelatine	8 g	8 g	8 g	8 g
Gum arabic	5 g	5 g	5 g	5 g
Salt mixture ³	7.2 g	5 g	5 g	5 g
l-cystine	0.1 g	0.1 g	0.1 g	0.1 g
Choline chloride	0.1 g	0.1 g	0.1 g	0.1 g
Cinchophen	0	0.5 g	1 g	0
Vitamin K substitute ⁴	1 mg	1 mg	1 mg	1 mg
d, l-alpha-tocopherol acetate ⁵		10 mg	10 mg	10 mg
Cod liver oil	5 g	5 g	5 g	5 g

For the *Gradation* ("scoring") of the symptoms it seems justifiable to use the scheme proposed by CHENEY (1942) in which Grade 0 represents no sign of ulceration, Grade 1: redness (fine hemorrhage) in the glandular layer, Grade 2: a break in the lining and beginning destruction of the glandular layer, Grade 3: a break in the lining, an ulcer with a flat base and indurated margins, and considerable destruction of the glandular layer, Grade 4: an ulcer extending into the muscular layer, and Grade 5: perforating ulcer. When several ulcers occur in the same gizzard, the most marked of them is used as an indicator for the severity of the symptom. Ulcers which finally perforate are usually found on or near the greater curvature of the gizzard near the zona intermedia. The bottom of the gizzard is seldom affected beyond grade 2. Ulcers are rarely found in the glandular stomach and none has been seen in the esophagus or in the duodenum in the present investigation.

CHENEY's scheme is based upon the assumption that the first signs of the defect is a beginning hemorrhage at the base of the glands which secrete the gizzard lining. The function of the glands is thereby disturbed or even stopped, so that the lining becomes weakened or even disappears over the affected areas. Then the gastric juice digests the underlying tissue, which increases the

¹ From S. M. A. Corporation, Chagrin Falls, Ohio.

² Fleischmann, Type 2019.

³ Consists of 7.16 g of McCollum's salt mixture no. 185, 0.996 mg KI, 9.93 mg CuSO₄ · 5 H₂O and 39.8 mg MnSO₄ · 4 H₂O.

⁴ Tetra-sodium-salt] of 2-methyl-1,4-naphthohydroquinonediphosphoric acid ("Sunkavite, Roche").

⁵ "Ephynal acetate, Roche".

depth of the ulcer. Changes may occur which apparently do not fit completely into this scheme. Thus a roughening of the lining may be found without evident changes in the glandular layer. Whether this is a manifestation of the same deficiency which may be the cause of ulcers is not absolutely certain. This stage has arbitrarily been counted as $\frac{1}{2}$. Rarely, a deep ulcer is seen which is not accompanied by gross damage of the lining.

In order to conserve time, the ulcers found in the present investigation were usually classified according to their gross appearance. Histological examination was used only in a few cases.

Feeding of the ulcer-producing diet to groups of 10 chicks for 4 or 5 weeks from the time of hatching produces the symptom to such a degree (average score for 10 animals: between 2 and 3) that comparison between test groups and a simultaneous negative control group can be carried out. Short feeding periods such as 1 or 2 weeks yield uncertain results.

A preliminary investigation has been carried out in order to determine whether a simple analysis of the gastric juice might replace the autopsy as a criterion for the tendency to ulcers.

The animals used in this investigation were reared from 18 to 62 days on the normal commercial diet, on artificial ulcer-producing diets with or without cinchophen, or on similar diets to which hog liver fat had been added. They had no access to food 24 hours before the test. The stomach juice was collected by means of French catheter no. 8 and a syringe, as described by CHENEY, with the modification that no sedative and no histamine were used. Several samples were taken at 10 minutes' intervals, and the pH was determined by means of the BECKMAN micro glass electrodes using two drops of each sample. For the animals on the normal diet the pH of the first sample varied from 1.6 to 2.1; that of the following samples differed from 1.4 to 1.9. For the animals on the various artificial diets the corresponding figures were 2.0 to 2.7, and 1.5 to 2.7 resp. The secretion from the chicks reared on the ulcer-producing diet often contained blood. The volume of the fasting secretion varied considerably in chicks of the same group (from 0.3 to 1.5 ml.). The difficulty in securing the optimal position of the catheter makes the figures for the volume much less accurate than those for the pH.

The conclusion from this limited investigation is that the fasting secretion of normal chicks is slightly more acid than the secretion of the chicks reared on the various artificial diets regardless whether they are ulcer-producing or not. It is therefore not feasible to use the pH determination as a criterion for the tendency to ulcers. The presence of blood in the fasting secretion may be suggestive. The acidity of the fasting secretion of the chicks on the ulcer-producing diets is not higher than that of the chicks receiving normal protective diets.

Results and Discussion of the Feeding Experiments.

Table 2 represents in condensed form the results obtained by adding various substances to the basal diets given in Table 1.

Table 2.

Section	Basal diet	Addition	Average ulcer-score in % of the ulcer-score in the corresponding negative control group
1	182	None	100
	182	2.5 mg % d,l-alpha-tocopherol acetate.....	53
	182	10 mg % d,l-alpha-tocopherol acetate.....	54
	182	lard instead of cod liver oil	25
	182	hog liver fat instead of cod liver oil	2
2	293	3.24 % "low calcium salt mixture" ¹ instead of 5 % of the original salt mixture	31
	293	ground whole wheat instead of starch	69
	293	ground yellow corn instead of starch	80
	293	ground oats instead of starch	93
	293	30 % of wheat bran instead of 30 % of starch	72
	293	15 % of frozen spinach mixed with the diet	89
	293	15 % of ventriculin ² instead of 15 % of starch	91
	293	0.5 % cholic acid	182
	293	10 % of cartilage ³ instead of 10 % of starch	51
	293	5 % chondroitin ⁴ instead of 5 % of starch	82
	293	25 % of pasteurized "20 %" cream mixed with the diet	61
	293	10 % of dried beef liver instead of starch	83
	293	10 % dried hog liver ⁵ instead of 10 % starch	32
3	203	10 % of dried calf brain ⁶ instead of 10 % of starch	39
	203	10 % hog liver fat instead of 10 % of starch	33
	203	10 % of lard instead of 10 % of starch	85
	203	2.5 % d,l methionine	88
	203	10 % of casein instead of 10 % of starch	77
	203	10 % of dried alfalfa	47
	203	10 % of cerophyl ⁷	76
	203	20 % canned sterilized "36 %" cream mixed with diet	104
	203	10 % of milk-sugar residue ⁸ instead of 10 % of starch	137

¹ Consists of the salt mixture indicated in footnote 3 of table 1 without the calcium lactate contained in McCollum's salt mixture.

² From Parke, Davis & Co., Detroit, Mich.

³ From the Wilson Laboratories, Chicago, Ill.

⁴ From The Armour Laboratories, Chicago, Ill.

⁵ From The Cerophyl Laboratories, Inc., Kansas City, Mo.

⁶ Furnished by S. M. A. Corporation, Chagrin Falls, Ohio.

Section	Basal diet	Addition	Average ulcer-score in % of the ulcer-score in the corresponding negative control group
3	203	10 % of dried hog liver ¹ instead of 10 % of starch	36
	203	20 % of dried hog liver instead of 20 % of starch	12
	203	18 % benzol-extracted dried hog liver ² corresponding to 20 % of dried hog liver, instead of 18 % of starch	52
	203	2.2 % benzol-extract of hog liver, ² corresponding to 20 % of dried hog liver, mixed with the diet	47
	203	5 % benzol-extract of hog liver ² instead of 5 % starch	50
	203	10 % benzol-extract of hog liver ² instead of 10 % starch	35
	203	10 % commercial soy bean oil instead of 10 % of starch	82
	203	0.84 % benzol-extract of alfalfa ² corresponding to 30 % of dried alfalfa	164
	293	5 % of hog liver fat instead of 5 % of starch	34
	293	5 % of hog liver fat instead of starch, mixture left on trays at 45-50° C before addition of cod liver oil	23
4	293	5 % of hog liver fat, heated to 100° C 1 hr, instead of 5 % of starch	31
	293	5 % of "peroxidized" hog liver fat ³ instead of 5 % starch	66
	293	2.5 % hog liver fat, instead of 2.5 % of starch	45
	293	2.5 % hog liver fat, autoclaved at 120° C 1 hr ² instead of 2.5 % of starch	99
	293	1 % acetone-insoluble fraction of hog liver fat	37
	293	same repeated	48
	293	4 % acetone-soluble fraction of hog liver fat	37
	293	1 % acetone-soluble fraction of hog liver fat	31
	293	5 % soy bean lecithin, prepared at low temperature ²	53
	293	5 % hog liver fat instead of 5 % of starch	17
	293	5 % hog liver fat instead of 5 % of starch + 0.5 % cholic acid	15
	293	5 % hog liver fat instead of starch	21
	293	3.8 % of fatty acids from hog liver fat ⁴	35
	293	0.8 % non-saponifiable from hog liver fat, corresponding to 5 % of hog liver fat ⁴	77

¹ From The Armour Laboratories, Chigaco, Ill.

² Furnished by S. M. A. Corporation, Chagrin Falls, Ohio.

³ 100 g of hog liver fat and 30 ml of 30 % H₂O₂ were dissolved in 1,000 ml of ether and left at room temperature for 24 hours, whereafter the ether was evaporated in vacuo and the residue taken up in petroleum ether. The peroxide value of the evaporated petrol ether solution was about 1,000 milliequivalents per 1,000 g.

⁴ The saponification was carried out at room temperature. 250 g of hog liver fat was dissolved in 625 ml of ether and mixed with a solution of 100 g KOH

Section	Basal diet	Addition	Average ulcer-score in % of the ulcer-score in the corresponding negative control group
4	293	5 % hog liver fatty acids, prepared by hot saponification ¹	22
	293	1.5 % fraction 1 of hog liver fatty acids ²	92
	293	1.5 % fraction 2 of hog liver fatty acids ³	75
	293	1.5 % fraction 3 of hog liver fatty acids ⁴	29
	293	1.5 % methyl-esters of fraction 3 ⁵	35
	293	2.5 % oleic acid instead of 2.5 % of starch	99
5	340	3.24 % "low calcium salt mixture" ⁶ instead of 5 % of the original salt mixture	39
	340	0.5 % cholic acid	88

The first section of Table 2 deals with the effect of modifying the basal diet 182, which is vitamin E free and does not contain cinchophen. It shows that the addition of vitamin E in the form of d,l-alpha-tocopherol acetate gives some but incomplete protection against ulcers. This observation brings to mind the statement of PAPPENHEIMER *et al.* (1939) according to whose work a deficiency of vitamin E affects the smooth muscle of turkey gizzards. It remains to be seen whether these two observations are related to each other. Substitution of lard for cod liver oil (with

in 625 ml of methanol. After 24 to 48 hours, 1 liter of ether and 1,650 ml of water was added. After separation and removal of the ether layer, the soap solution was again shaken out 8 or 9 times with 1 liter of ether. The combined ether-phases were rinsed with water, dried with sodium sulfate and evaporated *in vacuo* at 40° C. The soap solution freed from the non-saponifiable was mixed with 2,800 ml of water and 400 ml of ether and acidified with 150 ml of conc. hydrochloric acid. After separation the aqueous phase was again shaken with 1,400 ml of ether. The combined ether solutions of the fatty acids were rinsed, dried and evaporated in the same way as the nonsaponifiable.

¹ Refluxed on steam bath for 2 hours with potassium hydroxide in methanol, thereafter diluted with water, acidified with HCl, extracted with ether, rinsed with water, dried with Na₂SO₄, and evaporated *in vacuo*.

² Precipitated from a 10 % acetone solution at minus 20° C, iodine value 2.5 %, amount 37 % of total fatty acids.

³ Precipitated from filtrate from fraction 1 at minus 75° C, iodine value 90, amount 35 % of total fatty acids.

⁴ Filtrate from fraction 2 evaporated in vacuo, iodine value 210, amount 28 % of total fatty acids.

⁵ Prepared by refluxing the fatty acid fraction no. 3 on steam bath 2½ hrs. with twice its amount of methanol containing 1.3 % dry HCl, dilution with water, extraction with ether, rinsing with water, drying with Na₂SO₄, and evaporation *in vacuo*.

⁶ Consists of the salt mixture indicated in footnote 3 of table 1 without the calcium lactate contained in McCollum's salt mixture.

vitamins A and D supplied simultaneously in the form of concentrates) results in less marked ulcer production; hog liver fat provides a much better protection than lard.

The second section of table 2, dealing with diets with 1 % cinchophen and only 10 % cascin, shows that decreasing the content of calcium salts by removing the calcium lactate in McCollum's salt mixture 185, also results in less marked ulcer formation. The data do not permit the conclusion that the effect is specific for calcium salts, since lowering the other constituents of the salt mixture has not been tried. The effect is not confined to cinchophen diets (section 5 of table 2). BERG and ZUCKER (1944) have reported that calcium deficiency may lead to gastric lesions in rats, but this observation does not necessarily contradict the present finding, because the diets with lowered content of calcium salts still contain some calcium.

Section 2 of table 2 further reveals that cholic acid aggravates the symptoms, an effect which might be due to an increased absorption of cinchophen in the presence of added bile acid. When cholic acid is added to a similar diet without cinchophen (section 5 of table 2) there is practically no influence on the symptoms. The discrepancy between this finding and that of ALMQUIST (1938) perhaps can be explained on the assumption that the basal diets used in the present study are more completely deprived of factors which protect against gizzard ulcers.

Common cereals, such as wheat, oats and corn, give little or no protection against the ulcer-producing effect of cinchophen, even when they constitute a large percentage of the diet (60.3%). 15 % of frozen spinach and 15 % of ventriculin are also ineffective under the present circumstances, while some effect is obtained with cartilage and pasteurized cream; however, calf brain and hog liver are much more effective. A similar effect has not been obtained by the same amount of beef liver. The protecting effect of hog liver fat though considerable is less in chicks whose diet have cinchophen added than in chicks on diets which contain no cinchophen. The experiments with diets containing 0.5 % cinchophen (section 3 of table 2) show that canned sterilized cream and milk sugar residue gave no protection. Little protection is obtained with cerophyl¹ or by the addition of more cascin or me-

¹ Cerophyl is a commercial product consisting of the dried and powdered leaves of young cereal plants. CHENEY (1942) found it highly protective against gizzard ulcers.

thionine whereas dried alfalfa meal affords some protection. One of the most active substances is dried hog liver. Both the benzol-extract of hog liver and the benzol-extracted hog liver has furnished protection whereas the effect of commercial soy bean oil has been uncertain.

In section 4 of table 2 the protective factor in hog liver fat is studied further. This factor when mixed with the diet before the cod liver oil is added can withstand exposure to air at 40—50° C for one week. It can stand heating (in bulk) to 100° C for 1 hour whereas autoclaving at 120° C for 1 hour results in its destruction. Some loss occurs by treatment of the fat with hydrogen peroxide in ether. The acetone-soluble and the acetone-insoluble fractions do not differ greatly in potency, and both of these fractions are more active than soy-bean lecithin. The active substance of the fat is present in the fatty acid fraction, whereas the equivalent amount of the non-saponifiable substance has little effect. While in most of the experiments the saponification has been carried out at room temperature hot saponification with potassium hydroxide in methanol in a steam bath for 2 hours has not resulted in any appreciable loss of potency. Oleic acid has no effect.

The fact that hog liver fat and hog liver fatty acids protect against gizzard ulcer has already been suggested by certain experiments with vitamin K (DAM, 1935) which have been carried out both on a much smaller scale and during a considerably shorter duration than the study reported here. CHENEY (1942) also reported hog liver fat to be protective, but did not state that the effect is connected with the fatty acid fraction.

Using the method of BROWN and STONER (1937) the fatty acids from hog liver fat were divided into 3 fractions: viz. a practically saturated, a moderately unsaturated and a highly unsaturated (compare the footnotes to the corresponding part of table 2). Of these fractions, the practically saturated (iodine value 2.5) was nearly inactive, the moderately unsaturated (i. v. 90) slightly active, and the highly unsaturated (i. v. 210) very active. This latter fraction could be converted into methyl esters by heating with HCl in methanol without losing the potency.

Certain observations with other animals may be mentioned briefly:

When, for other purposes, ducklings were reared for 32 days on diets such as diet 182 with the modification that the cod liver oil in the diet had been heated to 100° C for a week with

air bubbling through it, the tendency to ulcers developed to a much higher degree than when the cod liver oil had not been heated. This observation recalls a report by MORRIS, LARSEN and LIPPINCOTT (1943) according to which prolonged feeding of heated lard to rats resulted in stomach ulcers.

Attempts to develop ulcers in rats by feeding artificial cinchophen containing diets similar to those used in the present studies on chicken gizzard ulcers failed to produce changes in the stomach within 94 days, even when 1 % of sulfasuxidine was added to the diet in order to reduce to a minimum the supply of nutritional factors from the intestinal flora. The fact that the stomach of the rat is more resistant to the deleterious effect of such diets may be related to the circumstance that the rat stomach does not secrete much acid during fasting, in contrast to the chick stomach.

The main result of the present study is that the highly unsaturated fraction of the fatty acids from hog liver, either as free acids or as methyl esters, counteracts the development of gizzard ulcers, although it does not result in a general improvement of the cinchophen poisoning. In addition it is possible to influence the tendency to ulcers by certain other modifications of the diet. It is worth while to notice that vitamin E and the factor in hog liver fatty acids act in the same direction, contrary to what is known about other effects of these two factors.

During the investigation here reported it was observed that the bill of chicks on the cinchophen and cod liver oil diets became affected in a peculiar way, namely, by erosion and formation of crust on the soft tissue of the upper bill. Eventually this change affected the bill itself to such an extent that in some cases the upper bill deteriorated completely. The lower bill was less involved. This symptom was influenced by certain modifications in the diet. Thus it was greatly lessened by feeding beef liver or methyl esters of the active fraction of hog liver fat; it was increased by feeding the corresponding free acids. The factor which protects the bill against the effect of cinchophen and cod liver oil is therefore not identical with the anti-ulcer factor.

Summary.

The highly unsaturated fraction of the fatty acids from hog liver fat (iodine value about 200) counteracts the development of gizzard ulcers in chicks reared on ulcer-producing artificial

diets. The moderately unsaturated fraction (i. v. 90) and the largely saturated fraction (i. v. 2.5) have little or no effect respectively. The active fraction can be converted into methyl esters without losing its potency and is relatively stable to heat.

Cod liver oil or oleic acid does not possess anti-ulcer effect; lard and commercial soy-bean oil are much less effective than hog liver fat.

Vitamin E in the form of d,l-alpha-tocopherol acetate delays to some extent the development of gizzard ulcers produced by an artificial diet without cinchophen.

The salt content of the diet also has some influence on the tendency to the development of ulcers.

Cholic acid is without effect when added to ulcer-producing diets without cinchophen but enhances the ulcer-producing effect of cinchophen-containing artificial diets.

Cartilage, pasteurized cream and dried alfalfa give some protection against gizzard ulcers. Dried calf brain offers a degree of protection somewhat comparable to that of hog liver.

Cerophyl, canned sterilized cream and ventriculin are largely inactive.

Evidence of increased acidity of the fasting stomach juice of chicks receiving ulcer-producing diets is not present.

Feeding of heated cod liver oil enhances the tendency to gizzard ulcers in ducklings.

Rats fed the same diets used for the production of ulcers fail to develop ulcers within 94 days.

Acknowledgement: This work was aided by a grant from Wyeth Incorporated of Philadelphia.

References.

ALMQVIST, H. J., Science 1938. 87. 538.
ALMQVIST, H. J., and E. L. R. STOKSTAD, Nature 1935. 136. 31.
ALMQVIST, H. J., and E. L. R. STOKSTAD, Ibidem 1936. 137. 581.
ALMQVIST, H. J., and E. L. R. STOKSTAD, J. Nutrit. 1937. 13. 339.
ALMQVIST, H. J., E. MECCARI, and F. H. KRATZER, Proc. Soc. Exp. Biol. N. Y. 1941. 47. 525.
BERGAND, B. N., and TH. F. ZUCKER, Bull. N. Y. Acad. Med. 1944. 20. 418.
BIRD, H. R., and J. J. OLESON, J. Biol. Chem. 1938. 123. xii.
BROWN, J. B., and G. STONER, J. Amer. Chem. Soc. 1937. 59. 3.
20—452546. *Acta phys. Scandinav.* Vol. 10.

CHENEY, G., Amer. J. Dig. Dis. 1938. 5. 104.
CHENEY, G., Proc. Soc. Exp. Biol. N. Y. 1940. 45. 190.
CHENEY, G., Arch. Intern. Med. 1942. 70. 532.
DAM, H., Nature 1934. 133. 909.
DAM, H., and F. SCHOENHEYDER, Biochem. J. 1934. 28. 1355.
DAM, H., J. Nutrit. 1944. 27. 193.
ESSELEN, W. B., Poultry Sci. 1939. 18. 201.
LANSING, A. I., D. MILLER, and H. W. TITUS, Ibidem 1939. 18. 475.
MORRIS, H. P., C. D. LARSEN, and S. W. LIPPINCOTT, J. Nat. Cancer Inst. 1943. 4. 285.
PAPPENHEIMER, A. M., M. GOETTSCH, and E. JUNGHERR, Bulletin 229. 1939. Connecticut State College, Storrs, Conn.
RABINOWITZ, M. A., J. Amer. Med. Ass. 1930. 95. 1228.
SCHWARTZ, S. O., and J. P. SIMONDS, Proc. Soc. Exp. Biol. N. Y. 1935. 32. 1133.
STALKER, L. K., J. L. BOLLMAN and F. C. MANN, Arch. Surg. 1937. 35. 290.
STOKSTAD, E. L. R., and P. D. V. MANNING, J. Biol. Chem. 1938. 125. 687.
WAGONER, F. H., and T. P. CHURCHILL, Arch. Pathol. 1932. 14. 860.

From the Rockefeller Laboratory of the Medical Clinic, Lund.

Studies on the Capacity of Serum to Bind Iron.

A contribution to our knowledge of the regulation mechanism of serum iron.

By

C. G. HOLMBERG and C.-B. LAURELL.

Received 30 August 1945.

Especially HEILMEYER and PLÖTNER (1937) have in fundamental works definitely shown that serum iron, *i. e.* the acid-soluble iron in blood serum, is a factor of physiological importance. We know that serum iron varies within fairly narrow limits in physiological conditions but the causative regulating factors are as yet practically unknown. This also holds for the factors which elicit changes in the concentration of serum iron in pathological conditions.

On the binding of serum iron.

Up to now all experiments indicate that serum iron is bound to one or more protein components in serum. BARKAN found (1927) that serum iron was nondialysable at the pH of the blood. He found, too (1933), that in native serum the serum iron is not ultrafiltrable. Only when the serum had been acidified did iron pass into the ultrafiltrate. VAHLQUIST (1941) showed that serum iron is nondialysable within a wide pH range from pH 4.5 to at least pH 10. VAHLQUIST's dialysis experiments *in vivo* also supported the view that serum iron is bound to proteins. BARKAN and SCHALE (1937) found that half-saturation with ammonium sulfate precipitated serum iron quantitatively together with the

globulins. Such was also observed to be the case with sera to which iron had been added (STARKENSTEIN and HARVALIK, 1933).

With the aid of modern cleftrophoresis technique VAHLQUIST (1941) has made a small series of experiments regarding the binding conditions of serum iron. His results are difficult to assess, as the iron concentrations in the analyzed fractions often were very low, the experiments were performed at different pH, and the iron concentration of the buffer employed varied. His conclusions are that serum iron is bound both to the albumin and the globulin fractions. The main part is found in the lastmentioned fractions, and one experiment seems to indicate that the α - and β -globulin fractions serve as the principal carriers, as the γ -globulin fraction is poor in iron.

In VAHLQUIST's monograph (1941) there is a review of the then accessible material regarding the normal variations of serum iron in man. He puts the mean values in men at $129 \gamma\%$ with $\delta 32$, and in women at $110 \gamma\%$ with $\delta 29$. From these figures it is naturally not possible to draw any conclusions as to the maximal capacity of native serum to bind iron. This point has, however, been elucidated in experiments with intravenous administration of iron. WALDENSTRÖM (1944) has published the hitherto greatest material on intravenous administration in healthy subjects and in various types of anaemia, where serum iron has been controlled continuously. In healthy subjects the mean value of the initial serum iron was $114 \gamma\%$ and 5 minutes after injection of 10 mg Fe^{+++} $291 \gamma\%$. WALDENSTRÖM draws attention to the interesting fact, earlier observed by SKOUGE (1939), that the concentrations of serum iron, obtained immediately (5 minutes) after an injection of iron, are lower than should be expected when calculating the quantity of iron supplied on the total plasma volume of the body. They are, further, considerably lower than the amounts of iron which can be bound in nondialysable form to the serum proteins in *in vitro* experiments. WALDENSTRÖM was able to preclude the possibility of this being due to the accumulation of iron in the blood corpuscles or to any loss of iron via the urinary tract, which corresponds completely with earlier investigations. (BARKAN, 1933, showed that blood corpuscles do not take up iron from sera to which iron has been added; HENRIQUES, ROLAND, 1928, McCHANCE, WIDDOWSON 1937, 1938, DAHL 1944 *et al.* all agree that only a very inconsiderable increase

in iron excretion in the urine takes place after intravenous iron administration.) After administration of iron the serum iron concentration obtained remains practically unchanged in healthy subjects for several hours. This "braking" of the increase in serum iron at intravenous administration often appears more clearly in patients with untreated pernicious anaemia than in healthy subjects. The "braking" may here be so strong that the initial serum iron value (fairly high in these cases) is scarcely influenced by the injection. The supplied iron in these cases exerts such a markedly toxic effect that the administration has to be discontinued long before the generally tolerated dose of 10 mg has been administered.

The deficient correspondence with earlier *in vitro* experiments, which showed a capacity of the serum to bind iron of quite another magnitude than that maximally obtained at intravenous administration of iron, immediately attracts attention. WALDENSTRÖM therefore does not seem inclined to discuss the possibility that the maximal values obtained in any way represent the upper limit to the binding capacity of serum. As he has proved definitely himself that the explanation cannot be sought for in an accumulation of iron in the formed elements of the blood or in excretion in the urine, the principal remaining possibility seems to him to be the influence of extravascular factors. He mentions in this connection the reticulo-endothelial system. He considers it possible that the extra-vascular factor may be subjected to hormonal regulation. The discussion of the braking effect is conducted in connection with the interpretation of the injection experiments in pernicious anaemia. In healthy subjects the "braking" is not very pronounced on administration of iron in doses up to 10 mg. He points out that cases of hepatitis do not often show any certain braking effect in spite of high initial serum iron values.

SKOUGE (1939) suspects that the "braking" is due to a protein precipitation of the iron.

In the experiments described below we have started with another hypothesis. We have reasoned with the possibility that the binding of iron which can be proved *in vitro* in sera to which large amounts of iron have been added is of a different character to the native iron-serum protein binding, and that the former therefore is easier dissolved *in vivo*, when the surplus iron is taken

up by tissue elements and thus disappears from the blood stream. Although this relationship has never been cleared up there are some hints in the literature to the effect that iron is bound differently in native serum and in such serum to which iron has been added respectively. The following early experiments show that such a thought is not altogether preposterous: When precipitating serum protein with trichloro-acetic acid the loss is perceptually smaller in sera to which iron has been added than in native sera (HEILMEYER, PLÖTNER 1937). VÄHLQUIST's electrophoresis experiments seemingly indicate that when iron has been added to a serum a greater percentage of it is bound to the albumin than is the case with native serum.

The primary condition of the hypothesis regarding the various types of iron-serum protein binding being provable experimentally is to have the disposal of a method which permits of determining at least one of these factors under physiological conditions. It is possible that if a substance which had the power of forming firm complexbinding with iron at physiological pH was added to serum, only the more loosely bound iron would be accessible to such a complex-binding, and that it would thus be possible to determine quantitatively the two hypothetical fractions *in vitro*. This substance ought further to give a strongly coloured complex with iron. If such a substance exists it would be feasible by means of adding iron *in vitro* to titrate the maximal limit of the native binding. In that case the boundary value ought to correspond to the values of serum iron, obtained after intravenous administration, *i. e.* the value at the onset of the braking. Thanks to a happy coincidence we have in $\alpha\alpha'$ -dipyridyl a substance which as far as we have found fairly well meets these requirements:

- a) It gives a strongly red-coloured complex within the pH-range 3.5—8.5 with ferrous iron (HILL, 1931).
- b) When adding dipyridyl to serum together with reduction agents no colour develops during the first hour. Even after several days only part of the iron has been liberated from its protein complex, which seems to be very poorly dissociated.
- c) When adding iron to serum a total complex formation between the colour reagent and the added iron is only obtained above a certain limiting value, special to each serum.

Methods.

Determination of total serum iron.

The method employed by us is an adaptation of the principles laid down by HILL (1931).

Test technique:

1. *Cleaning of glass ware.* After having been washed, all glass ware to be used for the analysis is treated with diluted hydrochloric acid and then rinsed with glass distilled water.

2. *Blood sampling:* About 20 ml blood is required. For taking the sample we used an inwardly polished needle of stainless steel, through which the blood flows down directly into a centrifuge tube (washed as described above).

3. *Analysis.* 3 ml 6N hydrochloric acid is added to 6 ml serum in a small erlenmeyer flask, is mixed well and left for 10 minutes, after which 6 ml trichloro-acetic acid is added. After another 10 minutes the mixture is filtered through a small Berzelius filtre¹ (the filtre papers should be washed in 5 % HCl and rinsed with glass distilled water and dried). The filtrate is collected in a graduated tube, holding 25 ml. 1 drop of paranitrophenol is added to 7.5 ml of the filtrate, and brought back almost to neutrality with ammonia, and acidified with N/2 hydrochloric acid until the colour has disappeared. After this 0.25 ml sodium acetate solution is added, and the sample acidified again with hydrochloric acid until the colour has disappeared (Obs. the acid should be added drop by drop). Some crystals of dipyridyl are added and about a knife's point of hydrosulfite. The solution is diluted with glass distilled water to 12.5 ml and well mixed. The extinction is determined in a pulfrich photometer against a blind which has been treated in the same way with glass distilled water instead of serum. The thickness of the layer should be 3 cm and the filtre S 50. It is very important to control that the sample does not contain any turbidity especially when the colour is very faint.

Computation:

The determined extinction multiplied by 955 = Fe in γ per 100 ml serum.

Solutions required:

6N hydrochloric acid (Conc. p. a. and equal parts of redistilled water).

20 % trichloro-acetic acid: The trichloro-acetic acid used had best be redistilled.

Conc. ammonia.

N/2 HCl: 5 ml conc. HCl diluted to 100 with redistilled water.

0.5 m sodium acetate: 81.6 g $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ is diluted to 1 l.

Paranitrophenol: 1 % solution in absolute alcohol.

$\alpha\alpha'$ -dipyridyl p. a. in substance.

Sodiumhydrosulfite: p. a. in substance.

¹ The observation made by VANOTTI and DELACHAUX (1942) that considerable amounts of iron are adsorbed by the filtre paper does not apply when using a Berzelius filtre No. 00 with a diameter of 7 cm.

Estimation of dipyridyl- and non-dipyridyl reacting iron in serum after addition of ferrous iron.

Principle: There is a certain boundary value for each serum below which added ferrous iron is firmly bound and does not react with dipyridyl, and above which all added ferrous iron reacts with dipyridyl. When determining the total amount of iron which may be firmly bound by a given serum it is therefore sufficient to make a determination after addition of so great an amount of ferrous iron that the total amount of iron in the sample surpasses satisfactorily the capacity of serum to hold iron firmly bound, henceforth called the "saturation limit".

The saturation limit is obtained by subtracting the value of the dipyridyl-reacting iron from the total amount of iron after administration of ferrous iron, both values being counted in $\gamma\%$. In the determinations here published we have, however, as a rule performed several estimations with additions of varying amounts of iron — at least 2 and generally 4 of which have surpassed the saturation limit — in order to achieve the greatest possible accuracy. The upper curve in figure 1 shows the results of a series of such estimations on a healthy subject.

Technique: For cleaning the glass ware see above on determination of total serum iron. About 40 ml blood is required.

Analysis: 6 ml serum is collected, and serum iron determined in the ordinary way as described above. 4 ml serum is pipetted into each of

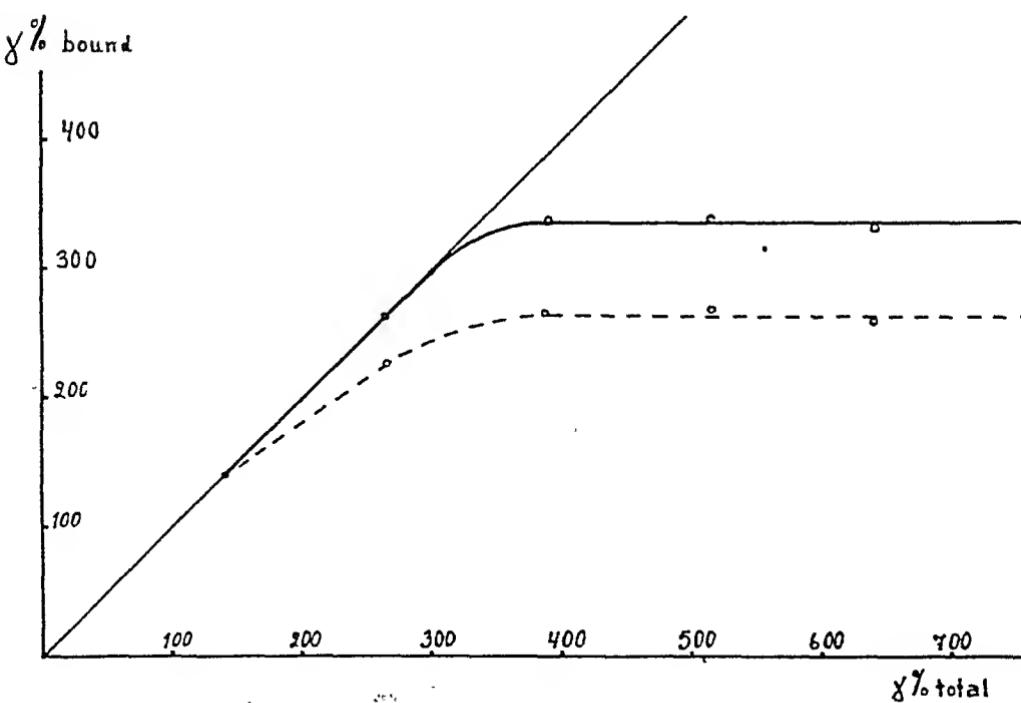


Figure 1.

two test tubes. 0.2 ml ferrous chloride solution is added to one tube, and 0.2 ml ascorbic acid solution to the other. The test tubes are filled with a mixture of 5 % carbon dioxide in nitrogen, and plugged. After 1 hour the samples are read off against each other in a pulfrich photometer with filtre S 50 in 1 cm cuvettes. The difference in extinction is noted (E_1). A knife's point of hydrosulfite is added to both tubes and some crystals of dipyridyl to the one containing iron. The tubes are filled again with the carbon dioxide mixture, and plugged. They are shook repeatedly for one hour. They are then read off once more against each other with the same filtre and size of layer as before (E_2).

$$\text{Computation: } \frac{100 [(\gamma/\text{ml} \cdot 4 + 20) - 6.88 \cdot (E_2 - E_1) \cdot 4.2]}{4} = \gamma \%$$

non-dipyridyl-reacting Fe.

Reagents:

Ferrous chloride solution: This is prepared as a stock solution of ferric chloride which contains 100 mg Fe and 1 ml conc. HCl per litre water. 10 ml stock solution is reduced with about 20 mg ascorbic acid immediately before being used.

Ascorbic acid solution: About 20 mg ascorbic acid is dissolved in 10 ml water.

Discussion of the method.

1. To convince ourselves that a complete development of colours takes place in the given conditions we performed some determinations in phosphate buffers of varying pH. The results are given in table 1. As the serum sample is kept under a gas mixture containing 5 % carbonic dioxide in nitrogen the pH remains fairly constant. Several measurements with the glass electrode have given values between 6.9 and 7.1. The time allotted to the development of colours therefore seems to be sufficient for all not firmly bound Fe present to be bound by the dipyridyl. We found, too, that extension of the time to 3—4 hours did not lead to any considerable increase in the colour strength.

Table 1.

The influence of pH and time on the complex formation of aa'-dipyridyl with iron.

pH	Extinction after 15 min.	Ex./40 min.	Ex./70 min.
5.85	317	318	316
6.50	313	314	317
6.80	311	314	314
7.52	306	314	316
8.00	300	306	315

2: When a ferrous iron solution containing a surplus of ascorbic acid is added to a serum a change of serum colours takes place. The colour of serum changes from yellow or yellow-green to yellow-red, and a definite increase in extinction can be measured in the pulfrich photometer with filter S 50. This change sets in successively on the addition of very small amounts of iron, and seems to attain its maximum at the saturation limit. If the saturation limit is exceeded, the extinction remains unchanged at maximum. It is as yet too early to pronounce with certainty on the basis of this change of colour. Several observations indicate, however, that it originates from the firmly bound iron in serum. Consequently we have as yet considered it justifiable to correct the extinction obtained with dipyridyl by subtracting this increase in extinction. If such a subtraction is not made it seems, when iron is added in amounts below the saturation limit, as if the iron did become only partially bound (see the dotted line in fig. 1). If the correction is made, we obtain a better-defined bend of the curve at the value for the saturation limit (see upper curve).

Saturation limit in healthy subjects.

We have determined the saturation limit in 10 healthy non-anaemic subjects (6 men and 4 women) by the methods described above. The results are given in table 2. Some more determinations

Table 2.

"Saturation limit" in 10 normal subjects after addition of increased amounts of Fe^{++} .

Case No.	Initial serum iron γ %	Saturation value γ %
1.....	160	264
2.....	160	366
3.....	140	335
4.....	172	303
5.....	138	336
6.....	110	266
7.....	84	358
8.....	140	261
9.....	132	298
10.....	80	335
Mean value: WALDENSTRÖM:	130 114	312 291 (intra-venous)

were made before the present technique was fully developed. These results are not included in the present paper, but the values obtained were of the same magnitude.

As seen from the table, these saturation values agree well with the maximum values attained by WALDENSTRÖM (1944) after intravenous administration of 10 mg iron.

We also made experiments with intravenous administration of 10 mg ferrous iron. We found that serum, collected within an hour after such an injection, is saturated — according to our definition — with iron. Directly after the injection we even found a small amount of dipyridyl-reacting iron. The total amount of iron is significantly smaller, however, than were to be expected on the assumption that the added quantity of iron is evenly distributed over the total serum volume.

Injection experiments with ascorbic acid reduced ferric chloride.

A healthy man, 25 years old. After 7 mg reddening of the face. At 9 mg nausea. 10 mg in all administered. Serum iron before injection 172 γ %. Saturation limit 320 γ %. Scarcely half an hour after injection serum iron 373 γ %, 325 γ % of which does not react with dipyridyl.

Injection of 10 mg on healthy man, 26 years old. At 9 mg sneezing. Serum collected immediately after injection. Serum iron 458 γ %. Saturation limit 410 γ %. Thus about 40 γ % reacted with dipyridyl. More serum collected after 90 minutes. Serum iron 420 γ %, which agrees with a saturation limit of 410 γ % within the limits of error of the method.

It seems therefore probable that the braking effect, observed by WALDENSTRÖM in injection experiments, might be explained by his having attained the saturation limit as determined in the present paper. We are of the opinion that the toxic symptoms which appear on injections with greater amounts of iron are due to this limits being exceeded, and to the fact that the surplus iron quickly leaves the blood stream and then exerts a toxic effect. In this connection there is no reason to assume a specific activity of the reticulo-endothelial system. The diffuse vascular reactions which set in most probably imply an effect on the capillary system, perhaps also on the central nervous system.

The correspondence with the braking effect discussed by WALDENSTRÖM is further illustrated in cases of pernicious anaemia, where this effect — as pointed out by WALDENSTRÖM — often is especially marked.

Saturation limit in pernicious anaemia.

WALDENSTRÖM's material shows that some untreated cases of pernicious anaemia show a nearly absolute braking effect, while in other cases serum iron may rise considerably after intravenous injection of iron. All liver-treated cases, on the other hand, show a marked increase in serum iron after intravenous injections. Table 3 gives the results of a series of determinations of the saturation limit in treated and untreated cases of pernicious anaemia and in one case of macrocytic anaemia of hepatogenic origin.

Table 3.

Saturation limit in untreated and treated cases of pernicious anaemias of a similar type.

Case No.	Serum iron γ %	Saturation limit γ %
Untreated, primarily saturated:		
Pernicious anaemia	1, 2, 3	170 255 246
" "	4	288
Hepatogenic macrocytic anaemia		
Pernicious anaemia of pregnancy		
Untreated, primarily non-saturated:		
Pernicious anaemia	5	156
Treated, non-saturated:		
Pernicious anaemia	6	20
" "	7	56
" "	1, 2, 2,	65 55
" "		210 170 250

It is seen from the table that 4 of the untreated cases were practically saturated, while one case was able to bind about 150 γ %. All the treated cases were able to bind relatively great amounts of iron before the saturation limit was reached. It is further seen from the table that the saturation limit does not change during the first days of liver therapy.

In 3 of the cases recorded in the table which proved to be saturated we tried to determine the braking effect *in vivo*, too. In all these cases toxic symptoms were obtained even after administration of very small amounts of iron: In two of these serum iron was determined directly after the injection. The values then obtained correspond evidently well with the saturation values. See case histories below.

Case histories.

Case No. 1. O. G. 60 years old. Woman. Since half a year increasing tiredness and loss of weight. On admittance haemoglobin 39, red blood count 1.6. Affected sensorium. Amnesia for the first week in hospital. Serum iron 170 γ %, primarily saturated.

Injection experiment with ferrous iron (reduced with ascorbic acid): After 1 mg nausea, coughing. At 2 mg feeling of heat in the face, blushing and increasing nausea. The injection was discontinued.

Case No. 2. O. R. 63 years old. Man. Since 2 months increasing tiredness. On admittance haemoglobin 36, red blood count 1.4. Serum iron 255 γ %. Saturation limit 260 γ %.

Injection experiment: After 1.5 mg violent sneezing. At 2 mg strong feeling of heat in the face. At 3 mg nausea. Injection discontinued. Serum iron tested after 90 minutes 264 γ %.

Case No. 3. E. J. 68 years old. Woman. Brother dead from osteomalacia hepatica. The patient has suffered from "muscle ache" and has had high sedimentation rate for several years. Macrocytic anaemia, not reacting on liver therapy, observed during the year of admittance. Pronounced diffuse osteoporosis. On admittance haemoglobin 56, red blood count 2.9. Sedimentation rate 113. Serum protein 8.4 (alb. 3.9, glob. 4.2 %). Sternal puncture shows nothing definitely pathologic. Serum iron 246 γ %. Saturation limit 260 γ %.

Injection experiment: After 2 mg "feels great heaviness in the eyes" and sees badly. Injection discontinued, but continued after 10—15 minutes up to 5 mg. Serum iron after one hour 265 γ %.

The investigated material of cases of pernicious anaemia seems to support the earlier made suggestion that the braking effect is an expression of the capacity of serum to bind iron firmly. The cases related above further seem to imply that transgression of this limit leads to the appearance of toxic symptoms.

Determination of the saturation limit in some other pathologic conditions.

We have also determined the saturation limit in some different pathologic conditions of various types, but the material is too small to allow any safe conclusions. The data from these investigations are given in table 4.

Two observations ought to be stressed, however:

1) The high saturation limit in the observed case of hepatitis acuta. As we know it has been observed earlier that some cases

Table 4.
Saturation limit in different pathologic conditions.

Diagnosis	Serum iron γ %	Saturation limit γ %
Polyarthr. chron. + anaemias	38	215
, , + , ,	15	215
Pneumonia + anaemia (fever)	64	180
Pleuritis + anaemia (subfebrile)	36	160
Anaemia (fever)	17	135
Hypertonias + anaemias	33	275
, + , ,	61	270
Diabetes + anaemia (epithelial symptoms)	15	250
Pregnancy with anaemia	30	385
Polyeytaemia rubra vera	40	420
Hypopharyngeal cancer with metastases	38	165
, , , , ,	56	260
, , , , ,	31	240
, , without , ,	84	230
Aplastic anaemia (ostitis fibrosa generalisata)	169	165
Acute hepatitis	256	690
Hereditary, non-haemolytic ieterus	103	396
, haemolytic ieterus	122	310

of hepatitis may show very high serum iron values after administration of iron.

2) The low saturation limit in patients with elevated temperature.

Summary.

By development of colour by means of dipyridyl in serum after addition of ferrous iron we have found that serum can contain about 300 γ % iron in a firm binding. The authors propose the term saturation limit of serum for iron for this figure. The braking effect discussed by WALDENSTRÖM after intravenous injections of iron seems to be due to the fact that the iron which is not firmly bound rapidly leaves the blood stream. The toxic effect at intravenous administration of iron sets in in connection with the transgression of the saturation limit when the surplus iron leaves the blood stream. In untreated cases of pernicious anaemia the iron content of serum is often so high that its capacity to keep the iron in firmly bound form is completely used up. These cases also react toxically even on 1—2 mg iron intravenously.

References.

BARKAN, G., Hoppe-Seyl. Z. 1927. 171. 194.
BARKAN, G., Ibidem 1933. 216. 1.
BARKAN, G., and O. SCHALES, Ibidem 1937. 248. 98.
DAHL, S., Nord. Med. 1944. 22. 835.
HEILMEYER, L., and K. PLÖTNER, Das Serumeisen und die Eisenmangelkrankheit, Jena 1937.
HENRIQUES, V., and H. ROLAND, Biochem. Z. 1928. 201. 479.
HILL, R., Proc. Roy. Soc. B. 1931. 107. 205.
McCHANCE, R. A., and E. M. WIDDOWSON, Lancet 1937. 233. 680.
McCHANCE, R. A., and E. M. WIDDOWSON, J. Physiol. 1938. 94. 148.
SKOUGE, F., Über das Serumeisen, Diss. Oslo 1939.
STARKENSTEIN, E., and Z. HARVALIK, Arch. exp. Path. Pharmak. 1933. 172. 74.
VAHLQUIST, B., Das Serumeisen, Diss. Uppsala 1941.
WALDENSTRÖM, J., (Järnbelastningar) Om Järn och järnterapi, Malmö 1944.
VANNOTTI, A., and A. DELACHAUX, Der Eisenstoffwechsel und seine klinische Bedeutung, Basel 1942.

From Universitetets Biokemiske Institut, Copenhagen.

On the Methylation of Guanidine Acetic Acid in the Animal Body.

By

GUNNAR STEENSHOLT.

Received 4 September 1945.

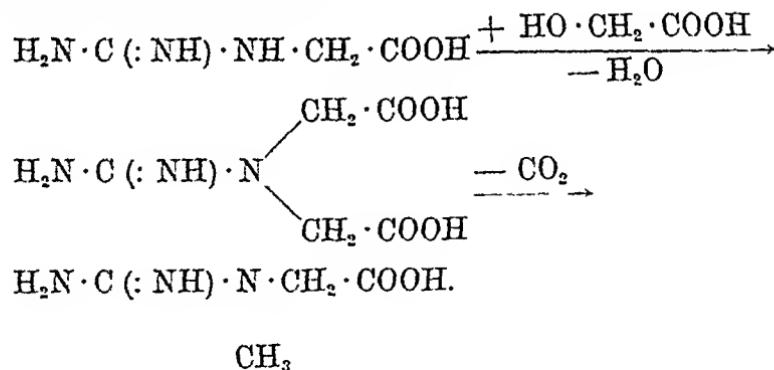
The problem of the synthesis of creatine in the animal body has occupied the attention of biochemists for a long time, and has been the object of a considerable number of investigations. The literature is therefore already far too extensive to be adequately reviewed in the present note. As regards the older and also much of the more recent literature on these questions reference is made to the well known and exhaustive treatise by GUGGENHEIM (1940). In the present note we must restrict ourselves to a very brief description of some of the main features of the problem as they present themselves today, particular attention being thereby paid to some of the more recent contributions.

BLOCH and SCHÖNHEIMER (1940) found that the guanidine group, probably enzymatically, can be transferred to other amino groups. In these experiments rats were kept on a diet containing arginine, in which the guanidine group was marked by ^{15}N , and glycine, in which the amino group was likewise marked by the nitrogen isotope. From the organs of these animals it proved possible to isolate creatine which contained the nitrogen isotope in the amidine as well as in the sarcosine part of the molecule. The highest content of ^{15}N in the creatine thus isolated was obtained on feeding ^{15}N -containing guanidine acetic acid. This latter compound may hence, when the well known structural formulae of the compounds in question are taken into consideration, be regarded as an intermediary in the biosynthesis of creatine.

This idea, which as a working hypothesis is not new (see GUGGENHEIM (1940)), together with some previous work on the methylation of ethanol amine by methionine in the presence of tissue slices (DU VIGNEAUD and associates (1940); STEENSHOLT (1945)) formed the starting point for the investigations which will be reported upon in the present note. The problem was to investigate whether guanidine acetic acid, in analogy with the biosynthesis of choline from ethanol amine, can be methylated to creatine in the presence of living cells or cell extracts, the methyl groups being furnished by methionine. An investigation along similar lines appears to have been carried out by BORSOOK and DUBNOFF (1940) (see below).

Mention must also be made of the work of LEHNARTZ and JENSEN (1941), and of MENNE (1941-42). They found that guanidine acetic acid, arginine and histidine by incubation with minced muscle can be converted into creatine. In particular MENNE made some interesting contributions to our knowledge of the enzymes or enzyme systems catalyzing the transformation in question.

A very interesting possibility has been pointed out by FISCHER, WILHELM and DAVENPORT (1938), and supported by some experimental evidence. According to these authors glycolic acid takes part in the methylation of guanidine acetic acid in the way indicated by the following scheme:



We shall finally recall some experiments by BARRENSCHEEN and PANY (1942), who found that etiolated wheat germs were able to convert guanidine acetic acid into creatine, and that the yield is greatly increased when methionine is added to the reaction mixture. BARRENSCHEEN and VALY (1942) also found that in the same way glycine, in the presence of methionine, can be transformed into betain.

The present work, the intentions of which were indicated above, had as its starting point the work of BLOCH and SCHÖNHEIMER (1940), and BARRENSCHEEN and his associates (1942), as well as the work on the methylation of ethanol amine referred to above (DU VIGNEAUD and associates (1940); STEENSHOLT (1945)). In the literature available to the present writer no reports of similar work have been found, except for a brief account of the work of BORSOOK and DUBNOFF (1940) alluded to above. However, these authors seem to have applied somewhat different methods. The present writer also believes to have collected some additional material which may be of interest to other workers in this field. It should also be borne in mind that, as is well known by students of this subject, the literature on the biosynthesis of creatine is crowded with conflicting results and views. It seems therefore that work by independent investigators ought to be welcomed.

Experimental Part.

Biological material. Organs (muscles and liver) from rats were used throughout. The animals were usually from 4 to 8 months old, and were kept on a diet believed to be sufficient in all respects. They were killed by decapitation, and the organs removed immediately after death. By means of a pair of bent scissors they were finely divided into a homogeneous mass, which could be conveniently handled and weighed.

Substrates. The guanidine acetic acid was synthesised according to NENCKI and SIEBER (1878), whose method was found to give satisfactory results. The methionine was a HOFFMANN-LA ROCHE product.

Determination of creatine. We have determined the amount of total creatinine in the reaction mixtures by the method of LIEB and ZACHERL (1934 a, 1934 b). The procedure was as follows:

2 ml of the deproteinated reaction mixture were pipetted into a 10 ml measuring flask. 1 ml n-hydrochloric acid was added and the mixture autoclaved for 20 minutes at 130° C. After cooling 0.4 ml 10 per cent NaOH and 4 ml saturated picric acid were added, and the mixture left standing for 10 minutes. Water was then added up to the 10 ml mark. Colorimetric measurements were then carried out with the Pulfrich photometer using filter S 53.

It is well known that the so-called JAFFE reaction, which forms the basis of the method just described, is not very specific. Thus guanidine acetic acid itself gives a colour which may to some degree interfere with the creatinine determinations by the LIEB and ZACHERL procedure. By working with suitable blanks, however, it is possible to eliminate the influence of interfering substances to a very large extent.

For deproteination a 10 % trichloroacetic acid solution was ordinarily used, but in all the experiments to be described below we also

carried out duplicate tests with a 50 % solution. In many cases the deproteinized reaction mixture had to be diluted with water (usually in the proportion 1 : 5) before measurement, in order to work in a convenient range on the photometer scale.

We first set out to establish experimentally the methylation of guanidino acetic acid with methionine acting as donator of methyl groups. A typical experiment was as follows:

The muscles from the hind legs of a rat were removed and carefully minced as described above. Into a small flask A were put

0.3 g minced muscle tissue,

15 mg guanidino acetic acid,

40 mg methionine,

4 ml phosphate buffer solution (pH 6.8).

A second flask B contained exactly the same amounts of tissue, guanidino acetic acid and phosphate buffer, but no methionine. Both flasks were incubated at 38° C for 12 hours. After this period 8 ml 10 % trichloroacetic acid solution were added to each of the flasks, and the contents stirred. After standing for 10 minutes the reaction mixture was spun down in the centrifuge. 2 ml of the deproteinized liquid were removed and used for the analysis of total creatinine as described above. Double analyses were always carried out.

We give in Table I the results for three different experiments of this kind. The table contains the direct photometer readings. In all three cases the reaction mixtures have been diluted with water in the ratio 1 : 5.

Table I.

	Flask A (Double analyses)		Flask B (Double analyses)	
1. experiment	1.86	1.90	1.51	1.56
2. experiment	1.79	1.80	1.52	1.49
3. experiment	1.81	1.83	1.49	1.52

(Each figure in the table is the mean of 5 independent readings.)

In these three experiments, chosen at random, the results were remarkably uniform, in spite of the fact that different animals were used in each experiment. In several further experiments of the same type, not reproduced here, the variations from one measurement to the other, *i. e.* from one animal to the other, were considerably greater.

Blank experiments were carried out by incubating methionine with minced tissue, and by mixing and incubating solutions of methionine and guanidino acetic acid. No increase in total creatinine could be found in the reaction mixtures.

In all this work the progress of the reaction was followed for periods of time varying from 5 up to 32 hours. The relative amounts of muscle tissue, guanidino acetic acid and methionine were also varied. However, the qualitative nature of the results always remained the same as in the cases reported above, *i. e.* a considerably higher total creatinine content in flask A than in flask B. We can therefore probably refrain from giving further numerical details.

When the above experiments were carried out with minced tissue which prior to the test had been heated to 70—80 °C, no increase in total creatinine could be observed.

Investigations along similar lines were also carried out in order to examine the influence of various buffers on the course of the reaction. Altogether the following buffers were tried

Borate — HCl (pH ~ 7.7)

Acetic acid — sodium acetate (pH ~ 6.1)

Veronal — acetate (pH ~ 6.5)

Ammonia — ammonium chloride (pH ~ 8.1)

Phosphate — borate (pH ~ 7.7)

Ordinary phosphate buffer (see above)

McIlvaine's phosphate-eitrare buffer (see below, in particular the diagram).

The reaction was found to work only in ordinary phosphate and in McIlvaine's phosphate-eitrare buffer.

In order to study the influence of the hydrogen ion concentration on the course of the reaction a series of experiments was carried out using McIlvaine's buffer. In a typical experiment flask A contained

0.3 g minced muscle tissue

8 mg guanidino acetic acid

30 mg methionine

5 ml buffer solution.

Flask B, of course, contained the same amounts of tissue, guanidino acetic acid, and buffer solution, but no methionine. The relative increase in total creatinine was computed from the experimental data, and expressed in per cent of the amount originally present. The quantity thus obtained was determined as a function of the hydrogen ion concentration of the reaction mixtures. The result is shown diagrammatically in fig. 1. Several other experiments of the same kind were carried out, but the numerical details are left out, since the diagram given can be

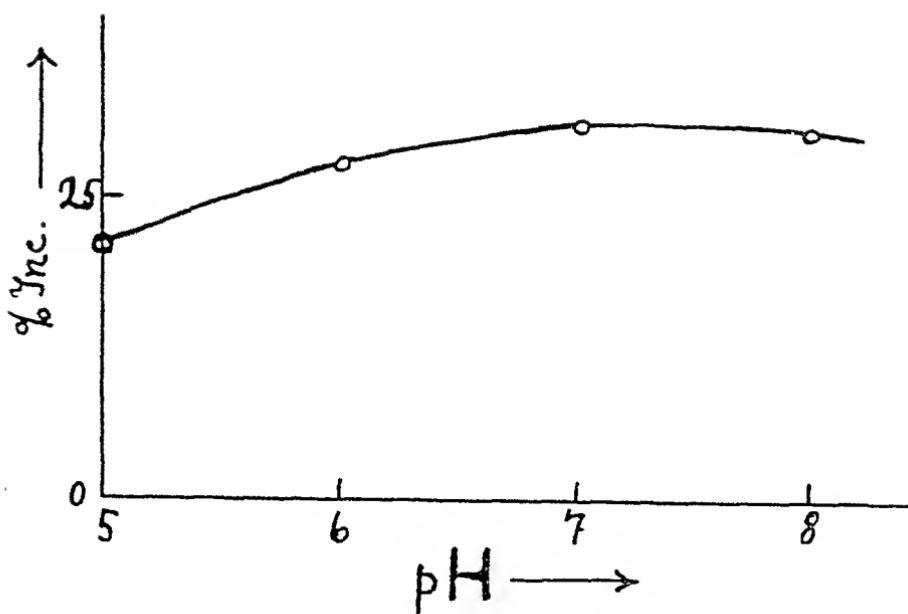


Fig. 1. pH dependence of the creatine synthesis. Abscissa: pH; ordinate: the relative increase (in per cent) of total creatinine in flask A.

regarded as sufficiently illustrative of the general nature of the results. The main feature is the fairly broad pH optimum somewhat above the neutral point.

Several attempts were made to obtain the enzyme or enzyme systems presumably responsible for the effects observed, in aqueous solution. These experiments were carried out by carefully mincing the tissue and then grinding it with sand in a mortar. Thus treated it was extracted with water, or with various salt and buffer solutions (physiological NaCl, Ringer solution, ordinary phosphate buffer, McIlvaine's phosphate-citrate buffer). The extraction periods lasted from 2 hours up to 2 days. The amount of solution used for the extraction was also systematically varied. Unfortunately, however, all efforts proved to be in vain the extracts were found to be inactive.

Finally the same set of experiments was carried through for liver tissue. The results were similar to those obtained for muscles, but the writer found that the increase in total creatinine brought about by incubating minced liver tissue with guanidino acetic acid and methionine was considerably smaller than for muscle.

Comments.

It can probably be safely concluded from the experiments reported above that muscle and liver in the rat contain enzymes capable of catalyzing the transfer of methyl groups from methionine to guanidino acetic acid, the latter substance thereby being transformed into creatine. It has the appearance that these enzymes are rather firmly bound to the cell structures; it must be remembered, however, that more efficient methods of extraction than those applied in the present piece of investigation may prove successful later on.

It would be interesting to know the explanation of the experimental result that the reaction seems to take place only in the presence of phosphate. However, a straightforward and unambiguous interpretation of the experimental findings is hardly possible at present; it may very well be that the failure in certain buffers is merely due to poisoning actions. Much more work is needed to clear up these obscurities.

The writer is glad to express his best thanks to Prof. R. EGE for his generous hospitality and support.

Summary.

It is shown by in vitro experiments that muscle and liver tissue from the rat are capable of catalyzing the methylation of guanidino acetic acid to creatine, the methyl groups being furnished by methionine. Some simple properties of the enzyme system in question are established.

References.

BARRENSCHEEN, H. K., and J. PANY, Biochem. Z. 1942. 310. 344.
BARRENSCHEEN, H. K., and T. VALY, Hoppe-Seyl. Z. 1942. 277. 97.
BLOCH, K., and R. SCHOENHEIMER, J. biol. chem. 1940. 134. 785.
BORSOOK, H., and J. W. DUBNOFF, Ibidem. 1940. 134. 635.
DAVENPORT, H. W., R. B. FISCHER, and A. E. WILHELMI, Ibidem. 1940. 132. 135.
GUGGENHEIM, E. A., Die biogenen Amine, Basel, New York 1940.
LEHNARTZ, E., and R. JENSEN, Hoppe-Seyl. Z. 1941. 271. 275.
STENSHOLT, G., Acta physiol. scand. (in press).
DU VIGNEAUD, V., J. P. CHANDLER, M. COHEN, and G. B. BROWN, J. biol. chem. 1940. 134. 787.

On the Action of some Adrenaline-like Substances on the Respiration of Suprarenal Tissue.

By

GUNNAR STEENSHOLT.

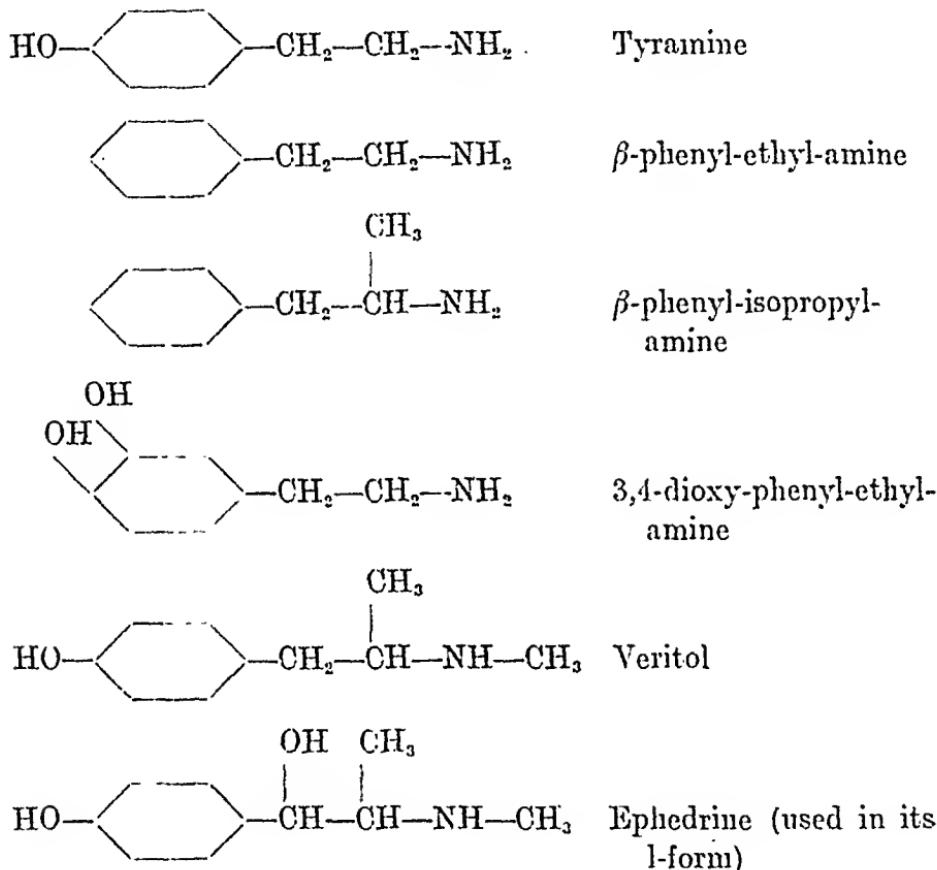
Received 4 September 1945.

Adrenaline is one of those hormones that are characterized by a relatively simple chemical structure, a fact which has considerably facilitated the study of its biological effects. It has therefore also been possible to study the physiological actions of a considerable number of substances of closely related type, namely those for which DALE has introduced the very descriptive term of sympathicomimetic compounds. These investigations are of interest since they may help to clear up the problem of functional groups in the adrenaline molecule. Further, they may give valuable clues to a solution of the very important problem of the biosynthesis of the hormone in question. Thus, it has been found (see, for instance, DEVINE (1940)) that tyramine, when incubated with suprarenal tissue, is transformed into adrenaline. Several other similar compounds have been found to exhibit the same property. Though the intermediary steps in this transformation have not been experimentally determined so far, the result is still obviously of great interest. We may finally mention that a further incentive to the study of sympathicomimetic substances is to be found in the circumstance that several of them have found rather extensive applications in practical medicine.

The foregoing remarks may serve as a partial justification for the present note, which arose out of an attempt to study the oxygen uptake accompanying the biosynthesis of adrenaline. During this work some observations were made, which form the subject

of this note, and which, in spite of their incomplete nature, may be of a certain interest in the further study of the suprarenals.

We give below, for reference, and in order to bring out the chemical relationships, the structural formulae of the adrenaline-like substances that were studied in the present work.



Experimental Part.

The aforementioned amines, the effect of which on the respiration of suprarenal tissue was to be investigated, were mostly used in the form of their hydrochlorides or hydrosulphates.

The respiration experiments to be discussed here were carried out as follows: Bull's suprarenals were used throughout. They were obtained fresh from the abattoire, where they were placed in an ice-cooled container immediately after having been removed from the newly killed animal. They were always used in the course of the next hour. A piece of tissue was cut out of either medulla or cortex, placed on a watch glass and very finely divided into a homogeneous mass by means of a pair of bent scissors. A suitable amount of this tissue mass

was then suspended in 2 ml bicarbonate Ringer in the main chamber of a Warburg vessel. The central chamber contained 0.2 ml of a 5 per cent potassium hydroxide solution for the absorption of carbon dioxide, and the side chamber contained 0.2 ml of an aqueous solution of the amine, the effect of which on the tissue was to be investigated. The vessels were placed in a water bath of 37.5° C. Temperature equilibrium was reached after 15—20 minutes, and the readings were then started. The respiration was followed for 30 minutes, readings being taken every 10th minute. At the end of this period the aqueous amine solution was tipped into the main chamber, and the respiration again followed for a suitable time.

The above procedure was also applied to certain other tissues, for instance liver, the technique being the same in all cases.

This way of studying the influence of substances on tissue respiration is simple and rapid, and has the advantage that both controls and tests are carried out on the same piece of tissue. It has previously been applied with success in studying the effect of various redox compounds on the respiration of animal tissue and bacteria (see, for instance, FRIEDHEIM (1934), STEENSHOLT (1945)).

In Table 1 we give, in as condensed form as possible, some examples of the results obtained by the above method for the effect of various sympathicomimetic amines on the oxygen uptake of suprarenal medulla tissue. We give in the table the oxygen uptake after the first 30 minutes' period, at the end of which the amine solution is tipped into the main chamber. In the next column we give the total oxygen uptake read off on the Warburg manometers at the end of another 30 minutes' period. In all the cases given in Table 1 the concentration of the amine solution in the side chamber of the Warburg vessel was around m/500.

Experiments were also carried out with 3—4-dioxy-phenyl-ethyl amine, but since this substance is very liable to autoxidation in aqueous solution, the results were not considered sufficiently clearcut to justify their being included in Table 1.

Table 1.

Effect of certain amines on oxygen uptake of suprarenal medulla tissue.

Amine	Oxygen uptake after 30 minutes in cmm	Oxygen uptake after 60 minutes in cmm
Tyramine	26.7	115.6
β-phenyl-ethyl amine	15.2	135.6
β-phenyl-isopropyl amine	23.0	137.1
Veritol	17.4	108.0
Ephedrine	10.0	95.3

A considerable number of similar experiments were carried out with each of the amines referred to above, thereby varying the concentrations of the amine solutions, the amount of suprarenal tissue, and also the length of the experiment. Double experiments were carried out, using bicarbonate Ringer in one and phosphate Ringer in the other series. The results were invariably the same in all the cases examined, and were, like the examples given in Table 1, characterized by a very marked increase in the oxygen uptake after addition of the amine solution.

Next an extensive series of blank experiments were carried out for each of the amines investigated, in order to see whether they are oxidized nonenzymatically in the presence of small amounts of adrenaline and whether the autoxidation of aqueous solutions of adrenaline is influenced by the presence of the amines. It is known that certain amino acids are nonenzymatically oxidized in the presence of small amounts of adrenaline (BLIX (1929)). Our experiments were carried out in exactly the same manner as the measurements with tissue slices, the tissue being replaced by adrenaline solutions. Various different relative concentrations of adrenaline and amine were tried for each of the amines, and several experiments were also always carried out with adrenaline solutions in which a part of the compound had been converted into adrenochrome by oxidation. However, in spite of all efforts, the writer was never able to demonstrate the slightest increase in oxygen uptake in these experiments after the amine solution had been tipped into the main chamber.

Several experiments were carried out to see whether for instance aqueous extracts of the suprarenal medulla showed the same effect as the medulla tissue itself. This was actually found to be the case. Thus, in one experiment, after thoroughly washing the finely cut and ground medulla tissue with a 0.9 per cent NaCl solution, the solution itself showed a very marked increase in oxygen uptake upon addition of an amine solution, as in the experiments referred to in Table 1. The washed tissue, however, did not show any effect.

Finally the amines under consideration were examined with respect to their effect on the oxygen uptake of tissue slices of liver, brain, kidney and muscle, mostly from rats. These experiments showed nothing unusual, and were found to be in complete agreement with existing knowledge of the properties and distribution of the enzyme monoamine-oxidase. The suprarenal cortex tissue behaved likewise.

Discussion.

In seeking an explanation for the effects described above our attention is naturally directed first towards the amine oxidases, and of course in particular the monoamine-oxidase. This enzyme is known to catalyse the oxidation of tyramine and β -phenyl-ethyl amine, and many other similar substrates. It can easily be shown to be present in the suprarenal medulla, for instance by using butyl amine as substrate. Repeated tests of this kind by the present writer actually demonstrated the presence of the enzyme. This fact furnishes an immediate explanation of the results described above in the experimental part of this paper as far as tyramine and β -phenyl-ethyl amine are concerned. However, as regards β -phenyl-isopropyl amine and l-ephedrine it is known that they are not attacked by the enzyme (BLASCHKO, RICHTER and SCHLOSSMANN (1937); verified by the present writer). It was further found during the present work that veritol is also not oxidized by the mono-amine oxidase. We are thus left with three compounds, which give rise to a very great increase in oxygen uptake in our experiments, though they are not attacked by the mono-amine oxidase.

It may be noted here that it is now pretty firmly established that tyraminase, adrenaline oxidase and aliphatic amine oxidase are identical enzymes; there seems very little room for doubt that we are dealing with a single monoamine oxidase.

The phenol oxidases form a group of widely distributed and fairly well-known enzymes, and it is very tempting to make some such enzyme responsible for the effects described above. It should be borne in mind, however, that a complete explanation is certainly not to be found in this direction. Phenol oxidases require the presence of at least one phenolic hydroxyl group in the substrate molecule, and this condition is certainly not fulfilled by β -phenylisopropyl amine. At any rate for this substance another explanation must be looked for.

We have already above, in the experimental part of this paper, drawn attention to the work of BLIX (1929), and pointed out the possibility of adrenaline acting as a nonenzymatic catalyst. However, explanations along these lines do not appear tenable in view of the blank experiments previously described. The same applies to adrenochrome and probably also to other oxidation products of adrenaline.

The outcome of the present discussion is, therefore, that for the time being no definite explanation of the effects observed can be put forward. As a tentative (and probably also very tempting) working hypothesis one would of course, with all the necessary reservation, like to advance the idea that we are dealing with new enzymes or enzyme systemis, capable of attacking the substrates in question. In this connection it is perhaps not out of place to recall some results for mescaline (3,4,5-trimethoxy-phenyl-ethyl amine), already described in the literature. BLASCHKO, RICHTER and SCHLOSSMANN (1937) found that this substrate is not attacked by mono-amine oxidase preparations from liver and intestine of guinea pigs. However, BERNHEIM and BERNHEIM (1938) found rabbit's liver to contain an enzyme capable of oxidizing the substance.

Further speculations along these lines seem idle at the present time, and must await the collection of further experimental material, whieh again is a matter for the future.

The author takes great pleasure in expressing his best thanks to Prof. EGE for his generous hospitality and support.

Summary.

It is shown that ephedrine, -phenyl-isopropyl amine and veritol cause a very marked increase in oxygen uptake of suprarenal medulla tissue. The possible explanations of the phenomenon are discussed.

Bibliography.

BERNHEIM, F., and M. C. BERNHEIM, J. biol. chem. 1938. *123*. 317.
BLASCHKO, H., D. RICHTER, and H. SCHLOSSMANN, Biochem. J. 1937.
31. 2187.
DEVINE, J., Ibidem 1940. *34*. 21.
FRIEDHEIM, E. A. H., Ibidem. 1934. *28*. 173.
STENSHOLT, G., Acta physiol. scand. (in press).

From Universitetets Biokemiske Institut, Copenhagen.

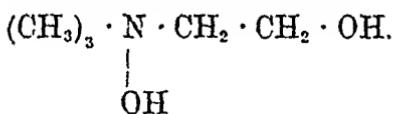
On the Synthesis of Choline in the Animal Body.

By

GUNNAR STEENSHOLT.

Received 4 September 1945.

Of the amines occurring in the animal body choline is one of particular interest to the biochemist. This is not only due to its rôle in metabolism, and the importance of the acetylated compound (acetylcholine) for the regulation of nervous processes, but also to the fact that choline contains a methylated amino group, as is evident from its structural formula

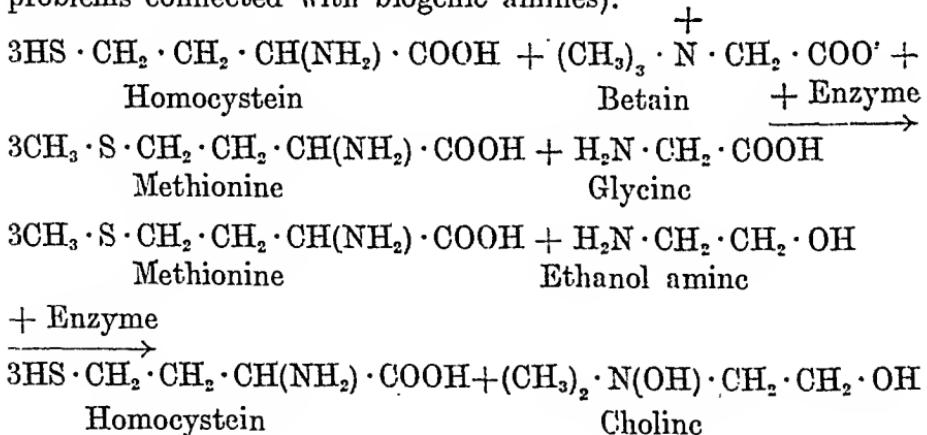


When the occurrence of substances of this kind in living beings had been established, the question immediately arose by which processes living cells were able to effect methylations of the type required for the synthesis of these compounds. The first to show that the animal organism is able to carry out methylation processes was His (1887). He fed pyridine to dogs and recovered it in the urine as methyl pyridinium hydroxide. However, the intermediary steps involved in these processes remained obscure; there was no experimental material available from which to decide what substances acted as donators of methyl groups, and the transfer mechanism likewise remained hidden.

A few years ago a change was brought about in this situation thanks to the work of DU VIGNEAUD, CHANDLER, COHEN and BROWN (1940), who have discovered a methylation process that may be of rather general importance. Their work may be briefly summarized as follows.

When rats are kept on a choline-free diet they show symptoms of avitaminosis. The growth ceases, and the animals exhibit the phenomenon of "fat liver" together with other changes in the normal metabolism. Choline is therefore a vitamin, and it may be concluded that the animal organism is not capable of direct methylation of amino ethanol, which is regarded as the precursor of choline (see BEST and HUNSMANN (1932), DE WITT (1941)) without certain dictetic requirements being fulfilled. The avitaminotic disturbances did not occur when methionine was fed instead of choline.

DU VIGNEAUD and his associates now fed methionine the methyl group of which contained heavy hydrogen, to rats, that were kept on a choline-free diet. From the organs of these animals it proved possible to isolate choline the methyl groups of which contained deuterium. It was also found that the symptoms of avitaminosis did not occur when the rats were kept on a diet containing no methionine, but homocystein and betain instead. From this it may be concluded that the methyl group of methionine can be transferred to the precursor of choline, and that homocystein receives methyl groups from betain and is thereby transformed into methionine. The whole process can be described as a transmethylation, and may be formulated as follows (see WERLE (1943), where an interesting review is given of this and several other problems connected with biogenic amines):



As is indicated in the above equations the transmethylation is probably catalysed by an enzyme or an enzymic complex.

All previous work on the methylation of amino ethanol seems to have been carried out on intact animals by the method of isotopes. It is probably not uninteresting to try to investigate the

same reactions by means of tissue slices or tissue extracts and more direct methods of choline determination. The writer has made some attempts in this direction, and the present note is a report on the results obtained so far.

Experimental Part.

Biological material. Rat liver or rat muscles were used throughout. The animals were from 3 to 5 months old, and had been kept on a diet believed to be sufficient in all respects. They were killed by decapitation and the organs to be used removed immediately after death.

Determination of choline. It was decided to apply a colorimetric method, since this is probably the more convenient procedure for series determinations. In the colorimetric determination of choline two methods are preferred:

- 1) Determination as choline iodide (ROMAN (1930));
- 2) determination of the choline reineckate (BEATTIE (1936)). Precipitation of choline by Reinecke salt is a method of considerable sensitivity, and is perhaps the most widely used method for this purpose. The determination is carried out by measuring colorimetrically the reddish colour imparted to acetone by choline reineckate. However, only relatively concentrated solutions furnish sufficient colour for accurate determinations. It may therefore be regarded as a considerable progress when ROSSI, MARENZI and LOBO (1942) studied photometrically the method for determining the chromate ion (CrO_4^{2-}) by a procedure based on the reaction of CAZENEUVE (CAZENEUVE (1900)), and found that it could be used for the determination of chromium in reineckates. On this basis MARENZI and CARDINI (1942) developed a new method for the determination of choline, which is claimed to be considerably more sensitive than those previously described. This procedure has therefore also been applied in the present work.

The technique is as follows:

Reagents.

Saturated solution of ammonium reineckate in distilled water, prepared at the time of using. The concentration of the solution is approximately 4 per cent.

96 per cent alcohol.

60 per cent acetone.

10 per cent NaOH.

10 per cent (by volume) sulphuric acid.

0.2 percent diphenyl carbazide in 96 per cent alcohol. This solution first acquires a faint rosy colour, which deepens after a few days. The solution can be used nevertheless, but the present writer has usually worked with solutions which were never allowed to become more than 4 to 5 days old.

The amount of choline to be determined varies between 15 and 100 γ . The volume of the sample may range from 1 to 3 ml. The sample is placed in a centrifuge tube with slender end and an equal volume of an aqueous solution of ammonium reineckate is added. The tubes are then placed in ice water for at least 20 minutes in order to complete the precipitation of choline. The writer has found, in agreement with MARENZI and CARDINI, that longer chilling does not affect the results. The precipitate is spun down in the centrifuge (4 minutes at 3,000 R. P. M.). At the end of the period of centrifugation the supernatant liquid is removed as completely as possible without loss of precipitate by means of a fine tube provided with a suction bulb. The precipitate is washed with 0.5 ml ice cold 96 per cent alcohol two or three times. The operation must be carried out with care lest some of the precipitate be lost.

The tubes are again chilled for a few minutes and centrifuged; the washings are repeated as described above. The supernatant liquid is now usually colourless, or almost so, but sometimes a third washing has proved necessary.

The precipitate is then dissolved in about 1 ml of acetone and the solution is transferred to an ordinary test tube. The centrifuge tube is rinsed 2 or 3 times with 1 ml 60 per cent acetone and the washings are collected and added to the solution in the test tube. We then add: 1 or 2 ml of water, 0.2 ml of sodium hydroxide and 0.1 ml of perhydrol for each 50 γ choline in the sample. Thus prepared the tube is placed in a boiling water bath. The heating is carried out carefully due to the rather rapid evaporation of acetone to begin with. After most of the acetone is evaporated the tubes are kept in the bath for 20 to 30 minutes. Finally they are heated over a naked flame for a few seconds to make sure the perhydrol is completely eliminated.

During the heating the liquid acquires a yellow colour. After the oxidation of the chromium the tubes are cooled and diluted with 3 or 4 ml of water. 2 ml of sulphuric acid are added together with sufficient diphenyl carbazide solution to give a final concentration of 8 per cent. The liquid is finally diluted to an appropriate volume in a suitable measuring flask, in our work to 25 ml. The photometric measurements were carried out with the Pulfrich photometer, using filter S-53. The comparison tube contained a blank consisting of 2 ml of sulphuric acid and 2 ml of diphenyl carbazide solution made up to a final volume of 25 ml.

A calibration curve is conveniently used.

Substrates. The methionine was a Hoffmann-la Roche product. The ethanol amine was partly a commercial product, carefully purified by repeated distillations, and partly synthesised by the writer according to KNORR (1897). Briefly described this method consists in leading a stream of ethylene oxide through a concentrated aqueous ammonia solution and subsequently fractionating the reaction mixture. The yield is unfortunately not very high, but was found satisfactory since only small amounts of the substance were needed in the present work.

Both samples were found to behave in the same way in all the experiments described below.

For deproteinization a 10 per cent trichloroacetic acid solution was ordinarily used, but in all the experiments to be described below we also carried out parallel experiments with a 35 per cent solution. In many cases the deproteinized reaction mixture had to be diluted with water (usually in the ratio 1 : 5) before analysis, in order to work in a convenient range on the photometer scale.

Our experiments first aimed at demonstrating the methylation of ethanol amine to choline in vitro. A typical experiment was carried out as follows:

The muscles from the hind legs of a rat were finely divided on a watch glass by means of a pair of bent scissors. If the mincing was carried out properly the quantitative handling of the tissue could be done with considerable accuracy. In a small flask A were placed

0.3 g minced muscle tissue

0.05 ml ethanol amine

40 mg methionine

4 ml McIlvaine's phosphate-citrate buffer solution (pH ~ 7.1).

A similar flask B contained exactly the same amounts of tissue and reagents, but no methionine. Both flasks were incubated at 38° C for 12 hours. At the end of this period 16 ml 10 per cent trichloroacetic acid were added to each flask and the contents stirred. After 30 minutes it was spun down in the centrifuge and 2 ml of the deproteinized liquid were removed for choline analysis according to the method of MARENZI and CARDINI. Double analyses were, as always, carried out. In table 1 below we give the results of three such experiments carried out on muscles from three different animals. The table gives directly the photometer readings.

Table 1.

	Flask A (Double analyses)		Flask B (Double analyses)	
1. experiment	0.43	0.44	0.37	0.37
2. experiment	0.59	0.60	0.45	0.46
3. experiment	0.55	0.56	0.40	0.41

(Each figure in the table is the mean of 5 independent readings.)

In several further experiments of the same type the results were qualitatively of the same nature, the relative increase of

the amount of choline in flask A being of the same order of magnitude in all cases.

A series of experiments of the same type were also carried out with minced liver tissue. We shall quote below only a single set of three experiments carried out on liver tissue from one and the same animal; this may serve to show the nature of the results for this kind of tissue and at the same time, together with table 1, give an idea of the accuracy of the work.

Flask A contained

0.5 g minced liver tissue

0.05 ml ethanol amine

40 mg methionine

4 ml McIlvaine's phosphate-citrate buffer ($\text{pH} \sim 7.1$).

As above flask B contained no methionine, but otherwise exactly the same amounts of tissue and reagents as flask A. The flasks were incubated for 12 hours at 38°C , and at the end of this period 10 ml 10 per cent trichloroacetic acid solution were added to each of them. After stirring, the mixtures were left standing for half an hour. They were then centrifuged and 2 ml of the liquid removed for choline analysis. The results are given in table 2.

Table 2.

	Flask A (Double analyses)	Flask B (Double analyses)
1. experiment	1.47	1.47
2. experiment	1.44	1.47
3. experiment	1.47	1.47

(Each figure in the table is the mean of 5 independent readings.)

The results of several other experiments of this type were of the same kind, and hence very similar to those obtained for muscle tissue.

It was found in separate experiments that the presence of methionine does not disturb the determination of choline by the method of MARENZI and CARDINI. Blank experiments were carried out by incubating methionine with minced muscle or liver tissue, and also by mixing and incubating solutions of methionine and ethanol amine. No choline synthesis could be demonstrated in these cases.

In all this work the course of the reaction was followed for periods of time varying from 4 up to 15 hours. The relative

amounts of muscle or liver tissue, ethanol amine and methionine were also systematically varied. However, since the results were qualitatively the same as in the cases reported above, showing a considerably higher amount of choline in flask A than in flask B, we shall refrain from burdening this report with the mass of all the numerical details.

If the minced tissue prior to the experiments had been heated to 70--80° C, no increase in the amount of choline could be observed.

Many experiments along analogous lines were carried out in order to investigate the influence of various buffers on the course of the reaction. The following buffers were examined

Borate — HCl (pH ~ 7.7)

Acetic acid -- sodium acetate (pH ~ 6.1)

Veronal — acetate (pH ~ 6.5)

Ammonia — ammonium chloride (pH ~ 8.1)

Phosphate — borate (pH ~ 7.7)

Ordinary phosphate buffer (pH ~ 7.0)

McIlvaine's phosphate-citrate buffer.

The reaction was found to work by far best in McIlvaine's buffer, but no definite evidence could be obtained from which to decide whether the presence of phosphate is necessary for the reaction.

In order to study the effect of the hydrogen ion concentration on the course of the reaction a series of experiments were carried out using McIlvaine's buffer. In a typical experiment flask A contained

0.3 g minced muscle tissue

0.05 ml ethanol amine

40 mg methionine

4 ml McIlvaine's buffer solution.

Flask B contained no methionine, but its content was otherwise identical with that of flask A. The relative increase in the amount of choline was computed from the experimental data, and the quantity thus obtained, expressed in per cent, was determined as a function of the hydrogen ion concentration. Fig. 1 gives the result in diagrammatical form. For liver tissue quite similar results were obtained. Further numerical details can probably be left out here. The main characteristic of the effect under examination, *i. e.* the pH optimum near the neutral point, is brought out sufficiently well in the diagram.

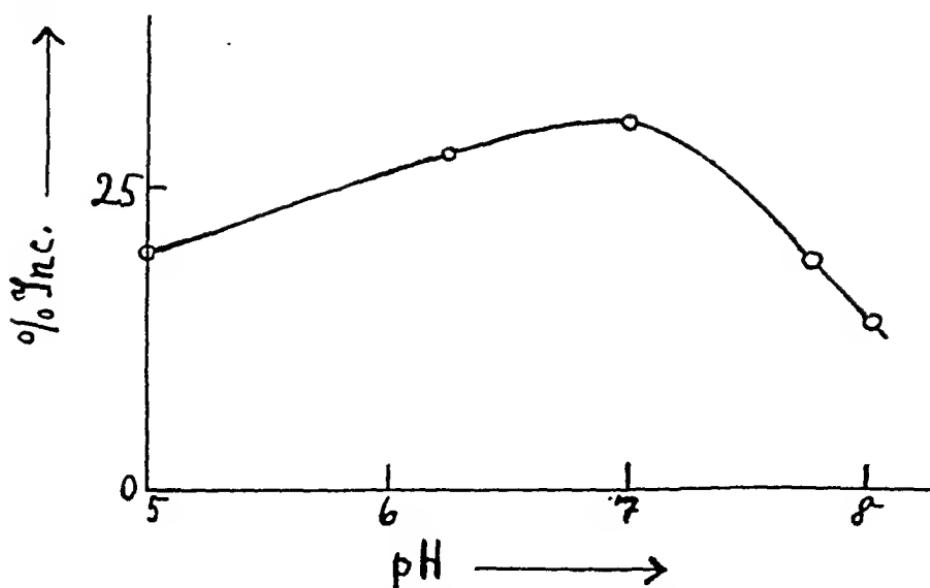


Fig. 1. pH dependence of the methylation of ethanol amine. Abscissa: pH; ordinate: relative increase (in per cent) of choline in flask A.

Finally several attempts were made to obtain the enzyme or enzymes presumably responsible for the effects observed, in aqueous solution. We carried out this work by carefully mincing and grinding the tissue with sand, and then extracting with water or aqueous salt or buffer solutions (for instance physiologic saline, Ringer, ordinary phosphate buffer, McIlvaine's buffer solution) for periods of time varying from 3 hours up to 2 days. The amount of solvent used for the extraction was also systematically varied. However, all extracts were found to be inactive; further numerical details are therefore of no interest. Both liver and muscle tissue gave identical results in this respect.

Discussion.

We are probably justified in drawing the conclusion from the experimental material presented above, that muscle and liver in the rat contain an enzyme or enzyme system capable of catalysing the transfer of methyl groups from methionine to ethanol amine, the ethanol amine being thereby converted into choline while methionine is demethylated to homocysteine. It seems at present that these enzymes are rather firmly bound to the cell structures, though of course the possibility remains that more

efficient methods of extraction than those applied in the present paper may later on prove successful.

The further work reported above does not call for much comment. Many problems concerning the enzyme systems in question still remain open, for instance the question of their distribution in the body and among the different species, the existence of activators and inhibitors et cetera. Their investigation must be left for future work.

The writer is glad to express his best thanks to Prof. R. EGE for his generous hospitality and support.

Summary.

From in vitro experiments evidence is gathered that muscle and liver tissue in the rat are able to catalyse the methylation of ethanol amine to choline, the methyl groups being furnished by methionine which is in turn demethylated to homocysteine. Some of the properties of the enzyme system in question are investigated.

References.

- BEATTIE, F. J. R., Biochem. J. 1936. *30*. 1554.
- BEST, C. H., and M. E. HUNTSMAN, J. Physiol. 1932. *75*. 405.
- CAZENEUVE, A., Analyst. 1900. *25*. 331.
- HIS, W., Arch. exp. Path. Pharm. etc. 1887. *22*. 253.
- KNORR, L., Ber. dtsch. chem. Ges. 1897. *30*. 909.
- MARENZI, A. D., and C. E. CARDINI, J. biol. chem. 1943. *147*. 363.
- ROMAN, W., Biochem. Z. 1930. *219*. 218.
- Rossi, L., A. D. MARENZI, and R. LOBO, An. farm. y bioquim. 1942. *13*. 1.
- DU VIGNEAUD, V., J. P. CHANDLER, M. COHN and G. B. BROWN, J. biol. chem. 1940. *134*. 787.
- WERLE, E., Die Chemie. 1943. *56*. 141.
- DE WITT, STETTEN jr., J. biol. chem. 1941. *140*. 143.

From the Rigshospital, Copenhagen, Department A.

A Pulse Time Recorder.

A Simple Apparatus for Recording Time Intervals as Ordinates.

By

SVEND JOHNSEN.

Received 4 September 1945.

In 1930 FLEISCH designed a pulse time recorder, an apparatus for recording as ordinates the time intervals between pulse beats. In principle and accuracy this device was so superior to all previous apparatuses that they need not be mentioned here.

In FLEISCH's pulse time recorder a lever recording on a drum is elevated at a constant speed by a motor. With the impact of the pulse the lever becomes electrically released from the motor, whereby it drops, to be instantly recoupled to the motor. Every pulse interval is registered as an ordinate corresponding to the height to which the lever is elevated. By means of various arrangements FLEISCH succeeded in recording every pulse interval with an accuracy of ± 0.004 sec.

The drawback of this instrument, however, is that it is extremely complicated and therefore expensive.

On the presupposition that in the course of physiological, pharmacological and clinical studies one desires more often to determine relative differences in pulse frequencies, when the experimental object is subjected to various influences, than absolute values, I have attempted to construct a more simple time ordinate recorder.

The Pulse Interrupter.

The time ordinate recorder requires that the oscillations whose frequency is to be determined can make and break an electric

circuit. In testing the pulse this is best obtained by employing FLEISCH's pulse pad. Only the following brief description need be given, interested readers being referred to the articles cited below.

A thin rubber membrane GM (fig. 1) over a small tambour PP is pressed outwards by a mercury column about 60 mm high in a narrow tube GK. When the tambour is placed upon the radial artery where it comes close to the radius, the membrane will be pressed in against the artery. Each pulse beat will move the mercury column, and, as the bore of the mercury tube is small (1.5 mm), the difference in the height of the mercury between systole and diastole is more than sufficient for securing contact with a platinum wire Pt which is inserted from above down into the tube. The average height of the mercury column must be constant all the time, and therefore it is in completely open connection (also during the recording) with an adjustable levelling bulb NG. This pulse pad acts satisfactorily, even with protracted recording.

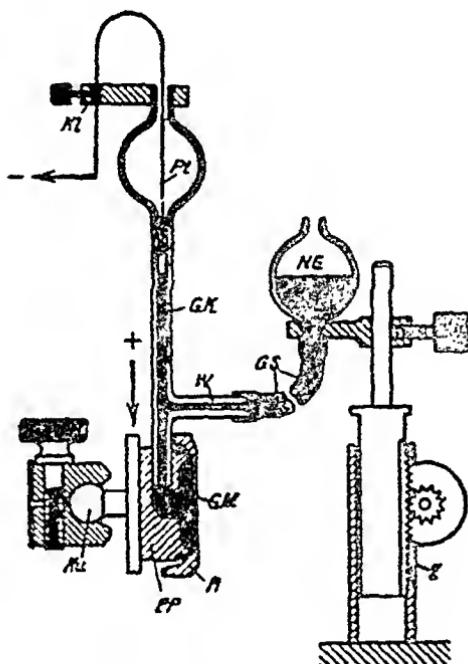


Fig. 1. From Abderhalden V, 8, page 916: FLEISCH's pulse pad. For description, see text.

The Time Ordinate Recorder.

Principle: A steady stream of air is fed into a Marey tambour. Each pulse beat must allow the air to escape again rapidly and completely. The length of the pulse interval determines to what degree the tambour is inflated.

Across a Marey tambour T (fig. 2) lies a lever V, which terminates in the recorder Sk. The tambour is connected to a metal T-tube R. Attached to the lower arm of the T-tube is a length of rubber tubing for the air feed. Soldered on to the T-tube is a casing containing a solenoid M with an iron core. Above the

solenoid is an armature K turning on the axis Ak and acting as a valve on the upper arm of the T-tube. The armature is elevated by a spring. The solenoid easing is mounted on a rod St, by means of which the entire apparatus can be attached to a stand.

If there is to be good proportionality between the length of the time interval and the ordinate, the air feed to the tambour must be steady and independent of whether the membrane is inflated or not. This is secured by feeding the air in at a pressure which is high compared with what is required to inflate the mem-

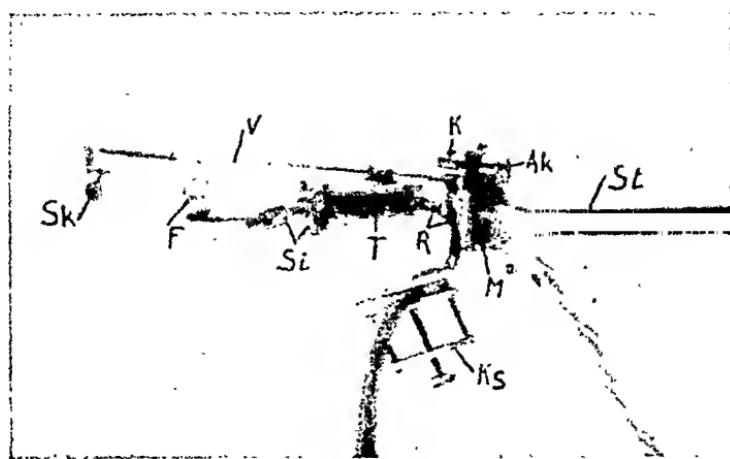


Fig. 2. Photographic reproduction of the time ordinate recorder.
For description, see text.

brane. Empirically, 1 atm. has been found to suffice. The easiest method is to employ a steel cylinder of compressed air with a reduction valve. Immediately in front of the tambour is the screw clamp Ks, which regulates the flow of air through the rubber tube. By this means the air in the reduction valve and the rubber tube does not form part of the air to be let out of the tambour; so the tambour is deflated more rapidly. The tambour must be fitted with a safety valve Si to avoid its bursting.

When a current is passed through the solenoid M the armature K is depressed, thereby closing the tube R; the tambour is inflated at a speed determined by the position of the screw clamp. When the current is shut off, the air escapes. By inflating the tambour membrane to a high tension, by making the internal capacity of the tambour small and giving ample dimensions to the T-tube, it is possible to make the tambour empty itself at a speed more than sufficient to register all pulse frequencies likely

varied somewhat from time to time. A really exact time-interrupter is difficult to make. Fig. 4 shows a curve drawn by the time ordinate recorder in connection with the metronome. The difference between the longest and the shortest ordinates is barely 3 per cent. of the entire length. It was impossible to decide whether this error was due to the metronome or to the time ordinate recorder. For the purpose of ascertaining the error over a longer period a curve was made extending over half an hour. The error did not exceed 6 per cent. The average ordinate in each minute

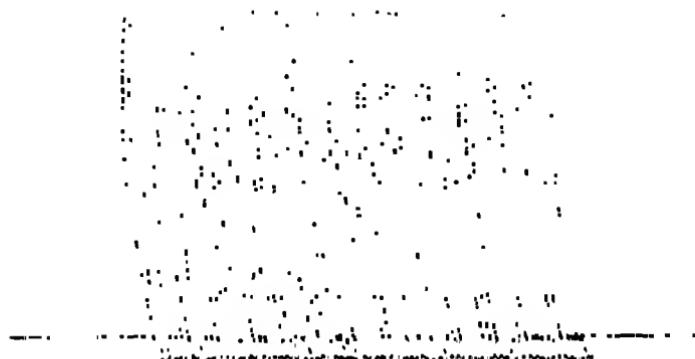


Fig. 4. Time curve when a metronome is used as the interrupter instead of the pulse pad. See text.

was constant throughout the whole experiment, from which it may be concluded that the air supply to the tambour was constant.

In longer experiments the average ordinate provides a correct expression of the time interval. On the other hand the length of each separate ordinate may have an error not exceeding ± 3 per cent.

Applications.

This time ordinate recorder was designed for pulse examinations on man.

It is one great advantage of the instrument, however, that it is capable of rapid adjustment to very different frequency ranges merely by regulating the air feed to the tambour. It can be used right from pulse examinations on small experimental animals (a reliable pulse interrupter for animal experiments is described by Tuusso and Tootson (1935)) to tests of the respiration frequency of man.

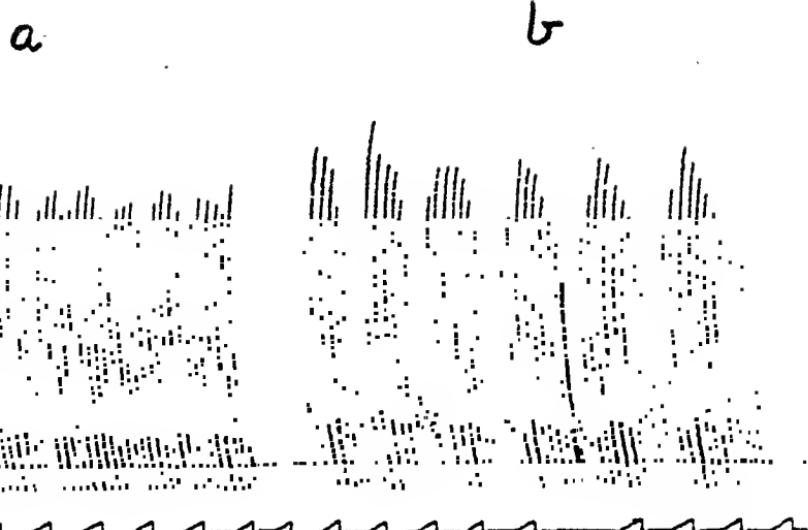


Fig. 5. Pulse time curve of a normal man with a: steady respiration, and b: arbitrarily deep respiration. Time reordering: 1/10th minute.

The air feed to the tambour not being fixed from one experiment to another, the length of the time intervals cannot be read directly from the ordinate. In most investigations where the object is merely to study relative frequency changes with various influences on the experimental individual, this is of no consequence. If absolute values are required, the instrument can be adjusted for each experiment in the following manner:

Immediately after drawing the time curve the recorder (without altering the air feed) is made to register on a rapidly rotating drum together with a time reordering of 1/10th or 1/100th sec. (determined by the frequency range to which the instrument is adjusted). Then when the current to the solenoid M is closed, the lever will rise at the same speed as during the experiment and record a long oblique line on the drum. From this line can be read the time interval corresponding to a certain ordinate. One drawback is that this must be done after each experiment, as one cannot rely upon the air feed being constant from one experiment to another (when the air cylinder has been shut off in the meantime, etc.).

Finally, Fig. 5 shows a pulse time curve made by a normal person with steady (a) and arbitrarily deep respiration (b). The respiratory arrhythmia is very manifest.

Summary.

The author describes a simple and inexpensive apparatus for recording time intervals as ordinates. The instrument, which is easily adjustable for widely different ranges of frequencies, is most suitable for the determination of relative frequency differences. When absolute time intervals are desired it must be calibrated after each experiment.

References.

ABDERHALDEN, E., Handb. Biol. Arb.-Meth. 8. 905.
FLEISCH, A., Z. ges. exp. Med. 1930. 72. 384.
TIITSO and Tootson, Pflüg. Arch. ges. Physiol. 1935. 236. 253.

From the Women's Clinic, Caroline Hospital, Stockholm.

Studies on the Functional Sphincter in the Cervix Uteri.

By

HANS FUCHS and AXEL WESTMAN.

Received 13 September 1945.

In a previous paper, one of us (WESTMAN, 1942) reported on a hitherto unknown functional sphincter mechanism in the cervix uteri. According to the degree of contraction of this sphincter, the superior part of the cervical canal is patent, constricted, or closed. If closed, the passage from the vagina to the uterine cavity is, of course, obstructed. Under certain conditions, it is possible to demonstrate that the muscles of the cervix work rhythmically.

No definite sphincter structure has been demonstrated anatomically. SCHRÖDER (1930) pointed out, however, that the superior portion of the cervical canal is narrower than the inferior portion. Below the internal os the cervical wall contains spiral muscular fibers, which form a marked ring directly under the mucosa.

In the present investigation the cervical sphincter is submitted to a more exhaustive study.

Method and material: In connection with laparotomy, in most cases performed because of sterility, a fine cannula was inserted through the wall of the fundus with its point projecting into the uterine cavity. The cannula was connected with an insufflation apparatus according to RUBIN, and the pressure was measured manometrically and also registered on a kymograph. Two specially constructed apparatuses were used alternately. The chief difference between them was that in one instrument the rate of

insufflation was about 350 cc. of carbon dioxide per minute and the kymograph rotated 37.5 mm. per minute, while the corresponding figures for the other instrument were 200 cc. and 14.5 mm.

Forty-six cases were examined in this way. In 45 of them menstruation was regular, and previous examination of the mucosa in the premenstrual phase had revealed a secretory phase.

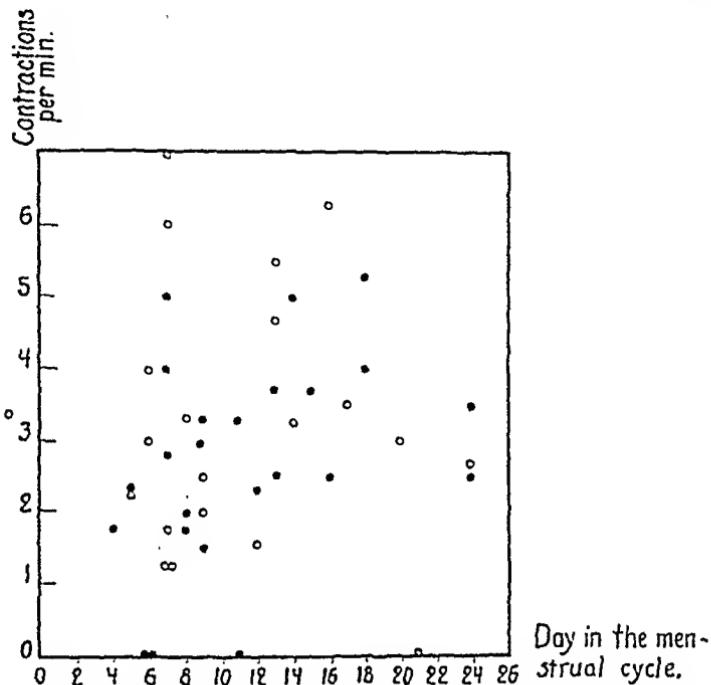


Fig. 1. Days in the cycle on which investigations were performed.

- Rate of insufflation: about 350 cc CO_2 per minute.
- Rate of insufflation: about 200 cc CO_2 per minute.

The remaining patient was amenorrhoeic. The uterus was grossly quite normal in all the cases. As will be seen from the diagram in Figure 1, the operation was performed in most of the cases between the fifth and the twentieth day of the cycle. In only three cases could the recording be done premenstrually, and in no case, of course, could it be done during menstruation.

On insufflation directly in the uterus, the pressure required to make the gas pass out into the vagina varies from case to case. This is due to the more or less tenacious clot of mucus in the cervix and partly also to the tension of the cervical muscles. An initial pressure of 100 mm. of mercury or more is often required to sweep away the mucus. The curve then drops, and the pressure

required thereafter ranged in the majority of the present cases between 30 and 75 mm. of mercury.

Under the experimental conditions described herein, the gas generally passes through the cervical canal in a rhythmic fashion, due to the intermittent contractions of the muscles forming the functional sphincter. When studying these fluctuations, consideration should be given to three main factors that may influence the passage of the gas: a) the anesthesia, b) the pressure in the uterine cavity and c) the rate of insufflation.

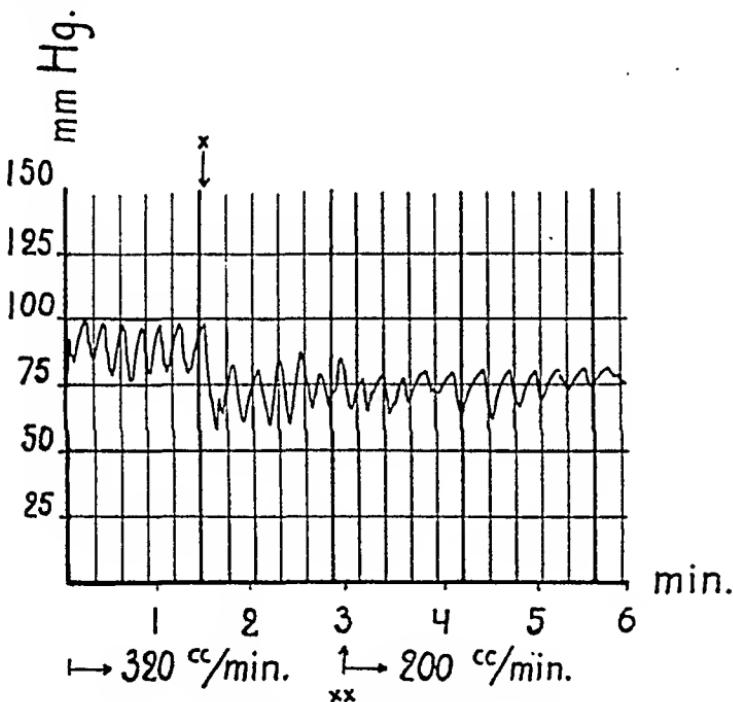


Fig. 2. Registration on the 15th day of the cycle.

a) With general anesthesia, the cervical musculature is relaxed, and the above-mentioned contractions are lacking. As soon as the patient begins to regain consciousness, however, contractions can be registered. The curve recorded on the kymograph then as a rule runs a very regular course. If the operation is done under spinal anesthesia, it will be noted that contractions can be seen from the very beginning.

b) The contractions in the cervical sphincter are not dependent on or definitely related to the gas pressure in the uterine cavity; this is true, at least, of the pressures in the present experiments, which never exceeded 150 mm. of mercury. This fact is illustrated in Figure 2. At the beginning of the experiment the pressure was

about 85 mm. of mercury. At X the cervix for some reason remained open a few seconds longer than usual. The intra-uterine pressure dropped, after which the curve exhibited a faint modification of rhythm at a gas pressure of 70 mm.

c) The rhythm is practically independent of the rate of insufflation used. This fact, too, is illustrated by Figure 2. At XX the insufflation rate was decreased from about 320 cc. a minute to about 200 cc. a minute. The frequency of the contractions was not changed appreciably, but the mean amplitude naturally decreased.

Results and Discussion:

Numerous observations on the function of the cervical sphincter both under spinal anesthesia and following section of the presacral nerve indicate that the sphincter is not under direct nervous control. It might therefore be assumed that it is the changes in the production of the ovarian hormone during the menstrual cycle that influence the muscles in question. In order to appraise the importance of the hormonal factors, it would naturally be desirable to make a series of examinations on one and the same case. Since this is out of the question, the only alternative is to compare cases examined on approximately the same day of the cycle. In this connection consideration was given to the frequency of the contractions.

In four cases the cervix was patent and the gas was able to pour freely out into the vagina the whole time the registration was taking place. Two of these were studied on the sixth day, one of them was studied on the eleventh and one on the twenty-first day of the cycle (Fig. 1). In all the other cases contractions were recorded. In general the frequency of the contractions was two to four per minute with a range of 1.5 to 7 per minute. As indicated by the diagram, there was no definite connection between the contraction frequency and the time of the menstrual cycle at which the recording was done. In some cases the curves at the beginning of the cycle exactly resemble those at the end of the cycle. The investigation thus provides no evidence that the function of the cervical muscles is directly controlled by the ovarian hormone. That such is not the case is also suggested by the fact that one case with amenorrhea exhibited a rhythm similar to that of certain other cases examined during both the first and the second half of the cycle.

The mechanism by which the cervical sphincter is regulated has not yet been determined. Studies are being conducted to determine whether certain chemical substances in the semen exert any influence.

It is, of course, highly probable that the muscular activity of the cervix is of the greatest importance to the transport of sperm. Experimental investigations indicate that this is the case in certain species of animals.

The normal contraction of the muscles that keeps the cervical canal closed for the most part is probably one of the protective measures aimed at blocking the passage of germs and the like to the internal genital organs and the abdominal cavity.

Summary.

In 46 cases carbon dioxide was insufflated during laparotomy into the uterine cavity through a narrow cannula passed through the uterine wall with the point projecting into the cavity. The experiments confirmed the observation made earlier by WESTMAN of a functional sphincter in the uterine cervix. When the cervix is patent the insufflated gas passes freely out into the vagina. In most of the cases the sphincter was closed and the passage through the cervical canal was blocked. Under the given experimental conditions a characteristic rhythm will be observed in the muscles of the cervix. This rhythm does not change in any typical manner during the different phases of the menstrual cycle. The biologic significance of the cervical sphincter is discussed.

References.

RUBIN, I. C., J. Amer. med. Ass. 1928. 90. 99.
SCHRÖDER, R., Handb. mikr. Anat. 1930. 7. 1.
Westman, A., Acta obst. gyn. scand. 1942. 22. 1.

From the Medical Department A of the Rigshospital, Copenhagen.

Bilirubin in Urine and Other Secretions apart from the Bile and in the Cerebrospinal and Eye Liquors.

By

TORBEN K. WITH.¹

Received 17 September 1945.

In jaundice when the bilirubin excretion with the bile does not take place to the normal extent the bilirubin is known under certain circumstances to appear in the urine. But while this phenomenon is comparatively well known our knowledge of its appearance in other secretions and excretions as well as in the body fluids is very limited, and the problems concerned are only summarily dealt with in the more recent literature. The bilirubin content of various secretions has, however, attracted new interest after it has been shown that the bilirubin of the serum is not freely diffusible but forms a stable compound with the serum albumin (BENNHOFF, 1932; PEDERSEN and WALDENSTRÖM, 1937). Theoretically the consequence of this protein ligation of the serum bilirubin would be that only secretions from glands with a special capacity for the excretion of bilirubin (loosening of the albumin-bilirubin ligation) could contain bilirubin, while all other secretions were free from bilirubin even in cases with severe jaundice.

In the following some observations on the occurrence of bilirubin in various secretions, excretions and body fluids are presented as well as some observations on the excretion of bilirubin through the kidneys.

¹ The studies presented here were aided by a grant from Frøken P. A. Brandt's Legat.

Previous Investigations.

The excretion of bilirubin with the urine has been studied by several authors while observations on the bilirubin content of other excretions and secretions are found almost exclusively in the older literature.

Urine: Most authors are of the opinion that bilirubin does not appear in the urine unless the bilirubin of the serum gives the so-called "direct" diazo reaction (HIJMANS VAN DEN BERGH). The hypothesis has been advanced that only bilirubin giving the direct diazo reaction can pass through the kidneys while indirect reacting bilirubin is unable to do so. In earlier papers (WITI, 1943; 1944) the author has, however, shown that the type of the diazo reaction of the serum bilirubin cannot be of primary importance to the excretion of the bilirubin with the urine, and that the serum bilirubin concentration is of considerably greater interest to the occurrence of bilirubinuria than the type of diazo reaction of the serum.

In man the excretion of bilirubin with the urine does only take place when the serum bilirubin concentration reaches a certain *threshold level*; in the dog, however, bilirubinuria occurs even with minute traces present in the serum, and various animals show great differences in this respect; but the behavior of bilirubin in different animals has been a field of study which has hitherto attracted only little attention.

HIJMANS VAN DEN BERGH (1918) found a threshold value of about 2 mg. bilirubin per 100 ml. serum in man, and several later investigators using the same analytical technique have arrived at similar results.

FALTITSCHER and HESS (1936) were able to demonstrate the absence of bilirubinuria in three cases of severe jaundice — serum bilirubin between 7 and 20 mg. per 100 ml. — suffering simultaneously from pronounced renal insufficiency and concluded that *the threshold value depends on the kidney function*. And as the serum bilirubin is protein bound and therefore unable to pass the glomerular filter it seems very likely that active kidney function must be essential for the occurrence of bilirubinuria.

That the human serum threshold shows considerable individual variation was shown by RETZLAFF (1923) and ANDREWES (1924). MEULENGRACHT (1920) found the threshold value at icterus index 40—50 which corresponds to 3—8 mg. bilirubin per 100 ml. serum according to the investigations of WITI (1942, a; 1943, a, b).

RABINOWITCH (1932) is of the opinion that bilirubin is often found as traces in the urine even when the serum concentration is normal, but the minute urinary concentrations found by this author are below the limit of accuracy of the existing analytical methods.

The only author who has hitherto carried out *simultaneous quantitative determinations of bilirubin in urine and serum* is RIESEL (1939) who used the hydrochloric acid method of HEILMEYER (1938) for the serum analyses and a modification of this method — including precipitation of the urine with $BaCl_2$ and colorimetric measurement of the

color produced by addition of concentrated hydrochloric acid — for the urine analyses. The result of his investigations is given in a diagram with columns from which it is seen that the urinary concentration of bilirubin was 10—30 % of the serum concentration in hepatitis (10 cases) while it was 40—90 % of the serum concentration in occlusive jaundice (9 cases); the absolute values of the concentrations are unfortunately not marked in the diagram.

In the newborn the threshold lies much higher than in the adult — about 18 mg. per 100 ml. serum — according to the investigations of LARSEN and WITH (1943).

Sweat: Some investigators have found small amounts of bilirubin (FRERICHS, 1858, p. 109; MÜLLER, 1887, p. 73; LEUBE, 1888) while others have found the sweat free from bilirubin even in intense jaundice (EPPINGER, 1937, p. 98).

Milk: According to FRERICHS (1858, p. 111) the milk of icteric mothers contains bilirubin, but UMBER (1926, p. 27) states that the milk of icteric mothers is bilirubin free.

All other secretions and excretions which have been subject to investigation — tears, saliva, gastric juice, enteric juice, pancreatic juice, vaginal secretion — are bilirubin-free according to all authors (FRERICHS, 1858; LEYDEN, 1866; MÜLLER, 1887; EPPINGER, 1937).

The bilirubin content of various body fluids is mentioned by several more recent authors.

Cerebrospinal Fluid: According to some authors the cerebrospinal fluid is always bilirubin-free even in the case of severe jaundice (BORCHARDT, 1923; SAIKI, 1931; EPPINGER, 1927, p. 98) while others have found small amounts of bilirubin — a few per cent of the serum concentration at the highest — in the cerebrospinal fluid in cases of jaundice of long duration (GÜNDELL and JACOBI, 1926; DE CASTRO, 1930); according to these authors the duration of the jaundice is significant to the occurrence of bilirubin in the cerebrospinal fluid as it is always absent in recent cases even of severe jaundice.

The Fluids of the Eye: The bilirubin content of the aqueous humor and the vitreous humor in persons suffering from jaundice does not seem to have been discussed in the literature. The so-called xanthopsia which occurs in some rather rare cases may be caused by bilirubin in these fluids but may also be due to other reasons — as bilirubin in the cornea or in the vessels or lymphatics of the retina.

Pure transudations are always bilirubin-free even in cases of severe jaundice, but inflammatory exudations as well as secretions from glands which are the seat of inflammation may contain bilirubin from the extravasation of blood caused by the inflammation (*cf.* EPPINGER, 1937, p. 98; BORCHARDT, 1923; SAIKI, 1931).

Author's Observations.

In the investigations presented here the serum analyses were performed with JENDRASSIK and GRÓF's (1938) method as modified by

the author (WITH, 1942 a, 1943 a). To urine and the protein-free secretions the method of the same authors for urine was used (*cf.* WITH, 1942 b).

Sweat, Tears, Saliva, Gastric Juice and Milk.

In ten patients with severe jaundice — four with occlusive jaundice, six with hepatitis — with serum bilirubin concentrations between 10 and 24 mg. per 100 ml. the sweat, tears, saliva and gastric juice were analysed for bilirubin. The secretion of sweat and saliva was provoked by injection of pilocarpine, the secretion of gastric juice by insulin injection and the secretion of tears by the nose-eye reflex (irritation of the mucous membrane of the nostrils by a small cotton swab). The sweat was collected by placing the patient on an oil-cloth from which a few drops were then collected in a test-tube. The tears were sucked up by small strips of filter paper placed in the eye corners; the bilirubin content was then estimated by the yellow color of the paper and its diazo reaction. The saliva was collected in a beaker and the gastric juice aspirated with a tube half an hour after the injection of the insulin. The bilirubin content of the secretions was estimated by their color as well as by their diazo reaction after addition of alcohol or caffeine mixture.

Not a single patient showed bilirubin in the above-mentioned secretions; *all the secretions were entirely bilirubin-free*. In the case of the sweat it is, however, to be emphasized that contamination with the bilirubin containing urine of the patients has to be avoided, which was accomplished by collecting the sweat only from the upper half of the body.

The examination of the bilirubin content of milk from icteric mothers was somewhat difficult to carry out owing to lack of material. In the Lying-in Departments of the Rigshospital only one case of jaundice in a puerperal woman occurred in the course of a year, and in this case the serum bilirubin concentration was only 3.19 mg. per 100 ml. As the renal threshold for bilirubin lies at 3—9 mg. per 100 ml. serum one could hardly expect to find bilirubin in the milk in this case, and consequently it says nothing about the excretion of bilirubin with the milk.

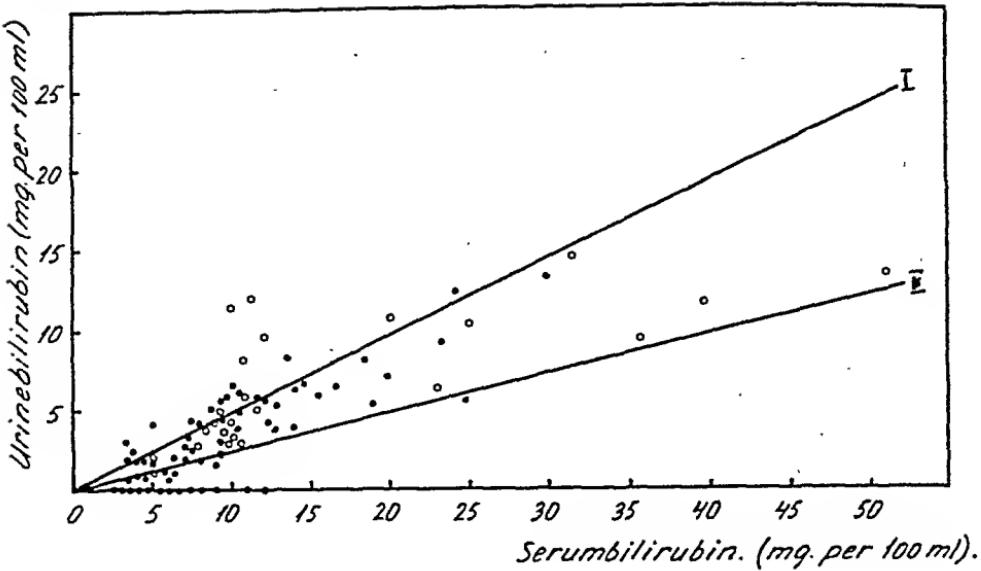
On the Excretion of Bilirubin with the Urine.

To determine the threshold value of the serum bilirubin concentration at which the excretion of bilirubin with the urine begins and to study the relation between the serum bilirubin and the urine bilirubin concentrations, simultaneous quantitative determi-

nations of serum bilirubin and urinary bilirubin were performed in 38 cases of acute hepatitis and 16 cases of occlusive jaundice. In several cases determinations were carried out twice or more as the jaundice varied in intensity during the period of observation, and a total number of 61 determinations were performed in the cases of hepatitis and 26 in the cases of occlusive jaundice. In all the determinations the urine was analysed within a few minutes after the urination in order to avoid losses from oxidation of the labile urinary bilirubin. The results of these analyses are seen from Diagram 1 in which the serum bilirubin concentration is plotted along the abscissa and the urinary bilirubin concentration along the ordinate (both in mg. per 100 ml.).

It is seen from Diagram 1 that the *threshold value most often varies around 5 mg. per 100 ml.*; the lowest value for the serum bilirubin by which bilirubin was demonstrable in the urine was 3 mg. per 100 ml. The author has analysed the urine for bilirubin in 25 cases of hepatitis with serum bilirubin concentration below 3 mg. per 100 ml. which are not included in the diagram, and in all these no bilirubin was demonstrable in the urine. When the serum bilirubin concentration is between 3 and 7 mg. per 100 ml. bilirubinuria may be present, but is in many cases absent. When the serum bilirubin is above 7 mg. per 100 ml. bilirubinuria is nearly always present, but in a few cases it is absent even with a serum bilirubin concentration of 7—12 mg. per 100 ml. In diagram 1 one case of chronic hepatitis with the serum concentration of 11 mg. per 100 ml. without bilirubinuria is included in which no signs of renal insufficiency were found, but the case of occlusive jaundice included in the diagram in which a serum concentration of 12 mg. per 100 ml. without bilirubinuria was found showed a pronounced renal insufficiency (blood urea concentration 330 mg. per 100 ml.); this patient suffered from a gall-stone in the common bile duct and chronic lymphogenous leukemia with pronounced leukemic infiltrations of the kidneys (found at autopsy).

As seen from Diagram 1 the *urinary concentration of bilirubin most often lies between 25 and 50 per cent of the concentration in the serum* (*cf.* the lines I and II drawn in the diagram: I corresponds to 50 % ($y = x/2$), II to 25 % ($y = x/4$)). The dispersion is, however, rather considerable and in a single case of occlusive jaundice (neoplasma of the head of pancreas in a 60-year-old man) the urinary concentration even exceeded the serum concentra-



- Occlusive jaundice.
- Hepatitis.

Diagram 1. The relation between serum and urine bilirubin.

tion. A systematic difference between the per cent of urinary excretion or the threshold value in hepatitis and in occlusive jaundice does not seem to exist — the very high urinary excretion per cent in a single case of occlusive jaundice (*cf.* above) is probably to be looked upon as accidental.

The interesting fact that the threshold value in the newborn (*icterus neonatorum*) lies at 18 mg. per 100 ml. according to the investigations of the author is mentioned above.

As renal insufficiency is known to impede the urinary excretion of bilirubin the idea suggested itself that the considerable variation of the renal threshold from case to case could be due to variations in the renal function, and the fact that the urinary excretion is not parallel to the serum bilirubin concentration even in one and the same case of jaundice could be explained in the same way. This explanation seems rather likely as hepatic diseases are known to influence the renal function under certain circumstances, the so-called hepato-renal syndrome (*cf.* NONNENBRUCH, 1941).

In order to demonstrate eventual relations between the degree of bilirubinuria and the renal function, simultaneous determinations of urea clearance and bilirubin clearance were carried out

on 10 icteric patients. The patients were given one liter of water to drink at the beginning of the clearance determination and observations were made during two successive periods of 60 minutes. The bilirubin clearances were calculated according to the common formula for clearance calculation (urine concentration divided by serum concentration and multiplied by the diuresis per minute) and the urea clearances were calculated as maximum clearances with a diuresis above 2 ml. per minute and as standard clearances with a diuresis below 2 ml. per minute.

The results are seen from Table 1. The bilirubin clearance values are seen to be very low — between 0.1 and 0.5 — indicating that *the ability of the kidneys for bilirubin excretion is very limited.*

Table 1.

Bilirubin- and urea clearances in 10 icteric patients.

Age	Sex	Diagnosis	Clearance period	Serum bilirubin (mg. per 100 ml.)	Urine bilirubin (mg. per 100 ml.)	Diuresis (ml. per minute)	Bili-rubin clearance	Urea clearance
42	♀	Chronic hepatitis	8—9 a. m. 9—10 a. m.	18.5 18.0	1.64 0.81	3.00 5.02	0.27 0.23	28 25
53	♀	Chole-lithiasis	8—9 a. m. 9—10 a. m. 10—11 a. m.	8.00 8.10 7.88	0.44 0.45 1.28	6.17 2.20 0.85	0.34 0.12 0.14	52 31 27
65	♀	Occlusion of common bile duct due to lymphogranulomatosis.	8—9 a. m. 9—10 a. m.	14.4 12.6	9.34 11.1	0.19 0.17	0.12 0.15	22 15
47	♀	Neoplasma of the pancreas	8—9 a. m. 9—10 a. m.	22.9 24.1	1.32 6.50	7.60 1.07	0.44 0.30	33 35
29	♂	Chole-lithiasis	9—10 a. m. 10—11 a. m.	7.13 7.54	3.46 2.42	1.08 1.20	0.54 0.39	65 72
27	♀	Acute hepatitis	8—9 a. m. 9—10 a. m.	19.1 17.0	0.88 0.22	1.95 8.00	0.09 0.13	19 28
23	♀	Acute hepatitis	8—9 a. m. 9—10 a. m.	11.4 11.3	2.35 0.42	1.08 3.75	0.23 0.14	30 47
22	♂	Acute hepatitis	8—9 a. m. 9—10 a. m.	8.40 8.03	0.54 0.33	2.20 4.67	0.14 0.18	50 69
32	♂	Acute hepatitis	8—9 a. m. 9—10 a. m.	9.58 7.98	1.67 0.48	0.90 5.42	0.12 0.34	49 68
34	♂	Chronic hepatitis	8—9 a. m. 9—10 a. m.	19.7 19.5	7.96 2.42	1.42 2.17	0.51 0.27	81 87

On other patients — 6 cases of hepatitis — clearance determinations were carried out during longer periods (3—4 hours) without giving water at the beginning of the clearance period (*i. e.*, with a much smaller diuresis); the results of these determinations are seen from Table 2. The table shows that the clearance may vary considerably from time to time in one and the same case.

Diagram 2 illustrates the correlation between bilirubin clearance and urea clearance; from Diagram 2 and Table 1 it is seen that there is no close correlation between the bilirubin clearance and the urea clearance, a circumstance going against the hypothesis that variations in the kidney function would be the cause of the variation of the renal threshold for bilirubin.

Diagram 3 illustrates the correlation between bilirubin clearance and diuresis and shows that the bilirubin clearance shows a certain tendency to increase parallel to the diuresis, a phenomenon which must be due to secretion of bilirubin by the tubular epithelia. For an excretion of bilirubin by ultrafiltration in the glomeruli cannot take place as the plasma bilirubin is firmly bound to serum albumin.

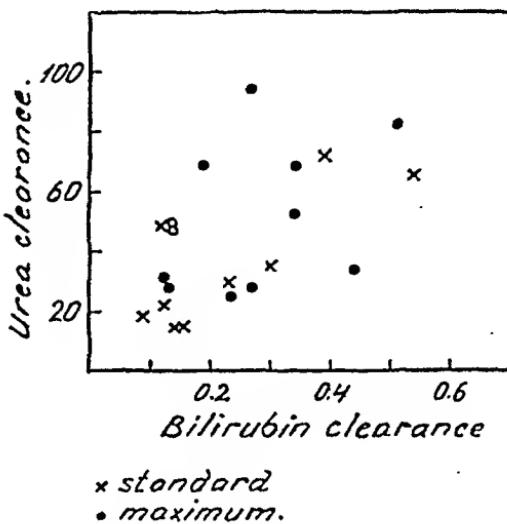


Diagram 2. Bilirubin and urea clearances.

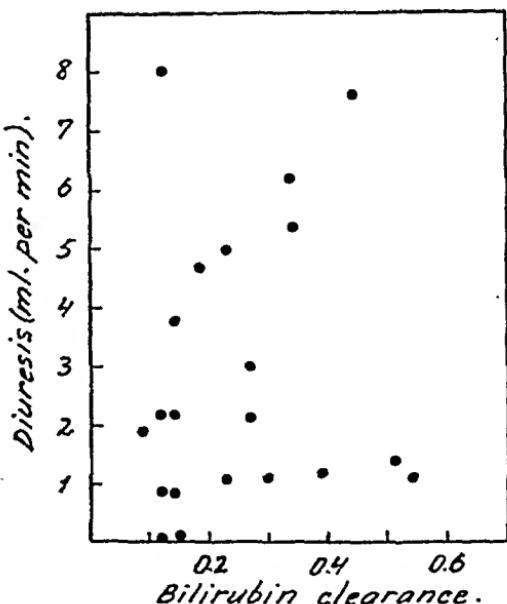


Diagram 3. Bilirubin clearances and diuresis.

Table 2.

Bilirubin clearance in periods of 3—4 hours in 6 cases of acute hepatitis.

Patient No.	Clearance period	Serum bilirubin (mg. per 100 ml.)	Urine bilirubin (mg. per 100 ml.)	Diuresis (ml. per minute)	Clearance
1	2½	12—15	9.43	5.64	1.38
1	2½	8—12	10.4	2.57	0.96
1	2½	12—15	10.4	2.26	1.06
2	2½	8—12	3.62	1.36	0.66
2	2½	12—8	5.42	1.11	2.14
3	2½	8—12	3.90	1.00	1.38
3	2½	12—15	3.90	1.28	1.40
4	2½	16—8	6.76	4.08	0.47
4	2½	8—12	6.16	2.90	0.58
4	2½	12—16	6.16	2.20	0.58
5	2½	8—12	7.10	1.80	0.66
5	2½	12—15	7.10	1.76	0.33
6	2½	9—12	9.40—10.4	3.04	0.58
					0.17

The Occurrence of Bilirubin in the Cerebrospinal Fluid and the Liquors of the Eye.

Lumbar puncture was performed on five icteric patients with serum bilirubin concentration between 10 and 24 mg. per 100 ml. In three of the cases the spinal fluid was entirely colorless while the other two showed a fluid of faint yellow color and a diazo reaction which was just visible and which on quantitative estimation corresponded to a bilirubin concentration below 0.1 mg. per 100 ml. So, the bilirubin concentration of the spinal fluid was at any rate below 1 per cent of the serum concentration in both these cases.

The bilirubin concentration in the aqueous and vitreous humors of the eye was determined post mortem in a case of neoplastic occlusion with a serum bilirubin concentration varying between 20 and 25 mg. per 100 ml. Both liquids were faintly yellow in color and showed a trace of diazo reaction corresponding to a bilirubin concentration below 0.1 mg. per 100 ml. This single observation seems to indicate that the humors of the eye like the cerebrospinal fluid are free from bilirubin in jaundice, with the exception of the occurrence of traces in cases of long-standing icterus.

This failure of the bilirubin to pass into the cerebrospinal fluid may be looked upon as a simple consequence of its ligation to

serum albumin and there is no reason to assume that it is due to some special function of the "blood-liquor barrier". The occurrence of minute amounts of bilirubin in jaundice of long duration cannot, on the other hand, be explained satisfactorily at present.

Summary.

In patients with pronounced jaundice the sweat, tears, saliva and gastric juice were found to be bilirubin-free. The behavior of milk in pronounced jaundice is yet unknown. The renal threshold for bilirubin is in the adult about 5 mg. per 100 ml. of serum (3—7 and occasionally as high as 11). The bilirubin clearance is very low (0.1—0.5) and does not show any close correlation to the urea clearance. The excretion of bilirubin in the kidneys seems to take place by secretion by the tubular epithelia only and the ability of the kidneys to excrete bilirubin is very limited. The individual variations of the renal threshold and the clearance for bilirubin are considerable.

The cerebrospinal and eye liquors are entirely bilirubin-free in most cases of jaundice. Only in cases of pronounced and long-standing jaundice may traces of bilirubin (below 1 % of the serum concentration) be found.

References.

ANDREWES, C. H., Quart. J. Med. 1924. 18. 191.
BENNHOLD, H., Ergebni. inn. Med. Kinderheilk. 1932. 42. 273.
BERGH, A. A. HIJMANS VAN DEN, Der Gallenfarbstoff im Blute; Leipzig, 1918.
BORCHARDT, H., Klin. Wschr. 1923. 2. 541.
CASTRO, U. DE., Dtsch Arch. klin. Med. 1931. 170. 176.
EPPINGER, H., Die Leberkrankheiten, Wien, 1937.
FALTITSCHEK, J. and I. HESS, Wien. Klin. Wschr. 1936. 49. 325.
FRERICHS, F. T., Klinik der Leberkrankheiten, Bd. 1. Braunschweig, 1858.
GÜNDELL, H. v., and J. JACOBI, Dtsch. Arch. klin. Med. 1926. 153. 215.
HEILMEYER, L., Biochem. Z. 1938. 296. 383.
JENDRASSIK, L., and P. GRÓF, Ibidem 1938. 296. 71 and 297. 81.
LARSEN, E. H., and T. K. WITH, Acta paediat. 1943. 31. 153.
LEYDEN, E., Beiträge zur Pathologie des Ikterus, Berlin 1866.
MEULENGRACHT, E., Dtsch. Arch. klin. Med. 1920. 132. 285.
MÜLLER, F., Z. klin. Med. 1887. 12. 45.

NONNENBRUCH, W., Dtsch. Arch. klin. Med. 1941. 187. 465.
PEDERSEN, K., and J. WALDENSTRÖM, Hoppe-Seyl. Z. 1937. 245. 152.
RABINOWITCH, I. M., J. Biol. Chem. 1932. 97. 163.
RETZLAFF, K., Z. ges. exp. Med. 1923. 34. 133.
RISSEL, E., Wien. klin. Wschr. 1939. 52. 873.
SAIKI, S., Jap. J. Gastroenterol. 1931. 3. 192, 195, 197 and 203.
UMBER, F., Handb. inn. Med. Berlin, 1926. 3. 2, 20.
WRTH, T. K., Nord. Med. 1942 (a). 13. 721.
WRTH, T. K., Hoppe-Seyl. Z. 1942 (b). 275. 166.
WRTH, T. K., Ibidem 1943 (a). 278. 120.
WRTH, T. K., Acta med. scand. 1943 (b). 114. 426.
WRTH, T. K., Nord. Med. 1945. 25. 505 and 26. 769.

From the Laboratory for the Theory of Gymnastics, University of Copenhagen.

A Method for Direct Measurement of the Aortic Pressure in the Dog.¹ (During Work on a Treadmill.)

By

ARNE P. SKOUBY.

Received 17 September 1945.

The method described in the following was developed from the arterial manometer of BUCHTHAL and WARBURG² (1943) utilizing in a similar way the capacity changes in a condenser, but suitably modified to record the pressures and pressure variations in the root of the aorta which are of special interest from the point of view of the circulation mechanism.

A flexible, but inelastic catheter (Fig. 1) is introduced through the carotid of the narcotized animal so that the tip is at the level

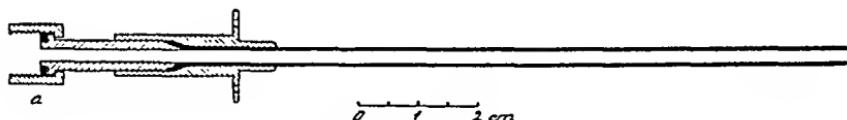


Fig. 1. Catheter to insert into the carotid of the animal. To the left a brass fitting for connection with the registering system by means of nut a.

of the opening of the brachiocephalic artery. The other end is fastened to the skin on the animal's neck, and when not in use the catheter is closed by a style. The catheter is a *Porgès catheter* no. 11 with an external diameter of 3.5 mm. It was found to be very resistant as compared with other materials tried out.

¹ The expenses of the work have been defrayed by the Frk. P. A. Brandts Legat foundation.

² For references c. f. this paper and E. WETTERER (1943). E. SCHÜTZ (1937) used the condenser principle for registration of pressure but no details have hitherto been published.

It is introduced under morphine (4—6 mg/kg intramuscularly $\frac{1}{2}$ — $\frac{3}{4}$ hour before the operation). A cut is made in the midline of the neck as distally as possible and the catheter, cut to a suitable length, introduced through the carotid and fixed by means of two silk sutures 1 cm from the opening. The most difficult point in the operation is to place the tip of the catheter just at the junction between the aorta and the brachiocephalic artery. The position should always be controlled by autopsy after the final experiment. The wound is moistened with body-warm salt solution until it can be closed and the brass plate sewed on to the skin. The wound is finally dressed with a plaster of Paris collar through which the tube protrudes freely. The plaster of Paris is changed each day. The morphine sleep is sufficiently deep for 1—2 hours and during the following hours the animal is distinctly affected, but the next day, or least the day after, the animal is completely normal and the catheter can remain in place for at least a week.

When an experiment is to be made the style is removed and the catheter connected with the glass tube (Fig. 2) by means of the

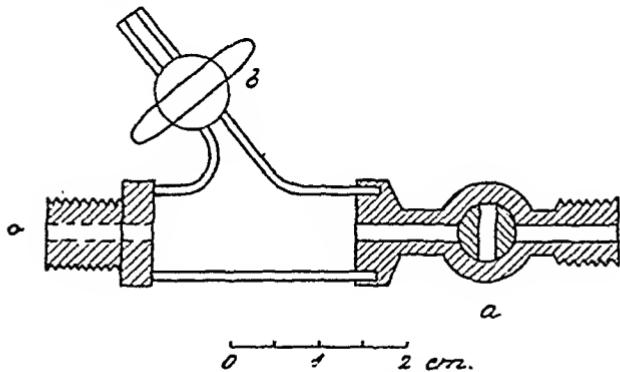


Fig. 2. Glass tube containing the pressure recording device during the experiments.
a) a brass tap. b) a glass tap. o) opening for the pressure chamber.

nut a (Fig. 1). This tube which is filled with sodium citrate solution can be opened also to the atmosphere through the glass tap (b). The pressure recording device (Fig. 3), manufactured in the laboratory workshop by Mr. Persson, is introduced into the glass tube through the opening (o) into which it makes an airtight fit. The lower end is triangular in cross section and is made up of a thin-walled phosphorbronze capsule containing an insulating body of the same shape and fitting snugly. Into the three faces of this body phosphorbronze plates are mounted so as to be insulated

from the capsule but connected together by a brass pin in the top of the insulating body. This arrangement, corresponding in principle to that adopted by BUCHTHAL and WARBURG, makes up a

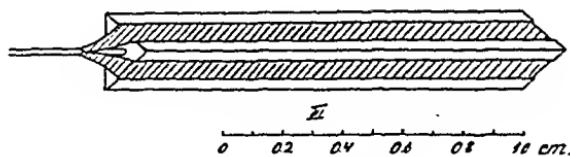


Fig. 3 a.

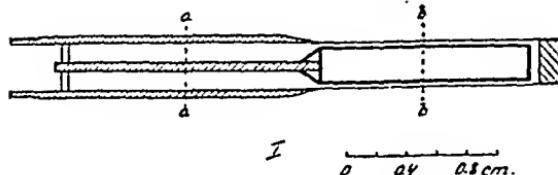
 Σ

Fig. 3 b.

Fig. 3. Pressure recording condenser.

I) capsule with insulating body. II) cross sections at a and b. III) inner body with phosphorbronze plates.

condenser the capacity of which is varied by the movement of the capsule walls when exposed to pressure.

The condenser is connected through a cable with a high frequency arrangement and this again with an oscillograph recording the pressure variations. Just behind the pressure recorder a silver tube (Fig. 4) is screwed on containing an autotransformer

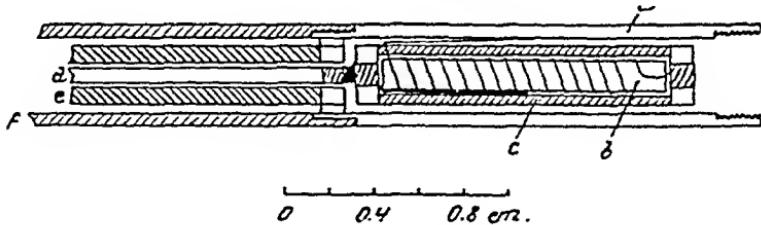


Fig. 4. Autotransformer.

a) silvertube. b) iron core of the transformer. c) iron shield of the transformer.
d) connecting wires. e) plastic material. f) copper tube.

for the purpose of reducing the effect of the cable capacity in relation to the capacity variations of the pressure recorder. The transformer is shielded by the silvertube to prevent a loss of the lines of force.

The cable is made of two parts, a proximal thin cable in permanent connection with the pressure recorder and the transformer

and a distal thick cable. The core of the thin cable consists of three copper wires and a piano string to take up any mechanical tension. The wires are insulated by plastic material and arranged in a copper tube.

The largest diameter of cable, silver tube and pressure recorder is 4.5 mm and the total length 40 cm. Rubber washers are put in between the various tubes.

The distal cable is an ordinary Siemens antenna cable which can be connected to the proximal by means of a revolving nut. This cable is 95 cm. long.

The condenser chamber with transformer and cable are part of an oscillating circuit (A) connected to the grid of a triode (Fig. 5).

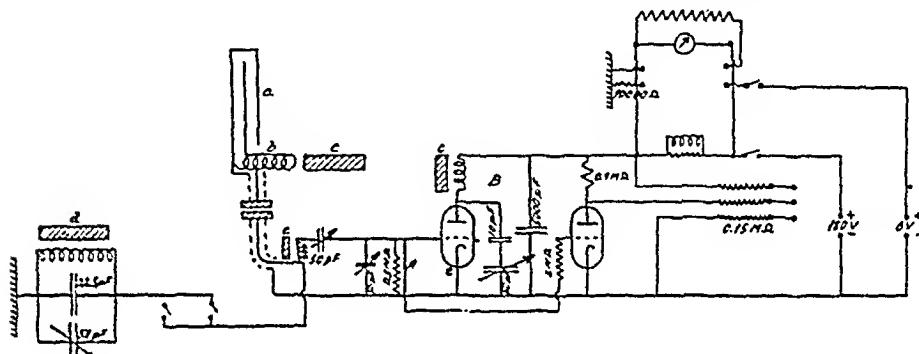


Fig. 5. High frequency circuit for converting pressure variations into variations of voltage.

A) oscillating circuit connected to the grid of the triode e. B) oscillating circuit connected to the plate of e.
a) condenser. b) autotransformer. c) ferrocard core. d) iron core.

This circuit is tuned to a resonance frequency which differs slightly from that of another oscillating circuit (B) connected to the plate of the same electronic valve. The triode produces high frequency oscillations, the necessary feed back between plate and grid circuits being obtained by the respective capacities. A change in pressure will alter the resonance frequency of the oscillating circuit (A) and increase or decrease the difference in resonance frequencies existing between circuits A and B. Thereby the feed back between plate and grid circuits and the intensity of the high frequency oscillations will vary with variations of pressure. The high frequency in the grid circuit are rectified and amplified by another triode the plate current of which is measured by a galvanometer used for coarse adjustment and by a torsion wire oscillograph. The deflections of the latter are recorded on photographic film.

paper. The high frequency device is constructed by Mr. P. F. BEER, Electrical Phonofilm, Copenhagen.

Control of sensitivity is obtained in two ways: 1. By replacing the condenser chamber, transformer and the proximal end of the cable by a capacity adjusted to the same size as these parts of the oscillating circuit and 2. by inserting a capacity the size of which corresponds to a definite change in pressure.

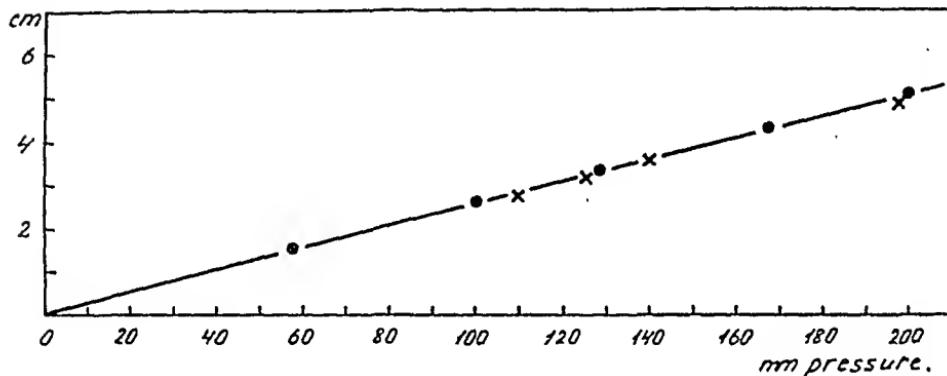


Fig. 6. Static calibration at 24° (—·—·—) and 34° (—×—×—×—) Celsius.

By introducing different static pressures the arrangement is calibrated *statically*. The capacity of the condenser chamber varies linearly with pressure changes in the range from 0—300 mm. Hg. It was furthermore ascertained that slow or sudden variations in

temperature are without measurable influence on the sensitivity of the pressure recording device (Fig. 6).

For *dynamic* calibration the pressure recorder is inserted in a tube, the other end of which is closed by the membrane of a condenser microphone and the entire system filled with oil (Fig. 7). The microphone membrane is operated by a laboratory oscillator and the periodically varying pressure

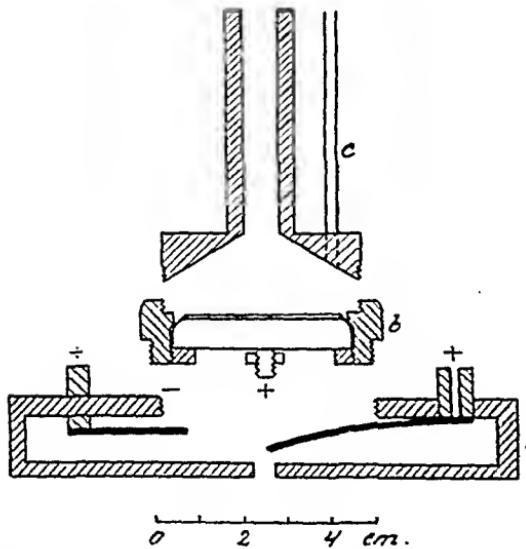


Fig. 7. Arrangement for dynamic calibration.
a) socle. b) condenser microphone. c) tube for inserting of the pressure recorder.

amplitudes are recorded with an A. C. amplifier and oscillograph for different oscillation frequencies. The system reacts to frequencies from 0—300 cycles per sec. the pressure amplitude being approximately constant between 0—100 cycles per sec. Higher frequencies are registered with diminished sensitivity (Fig. 8).

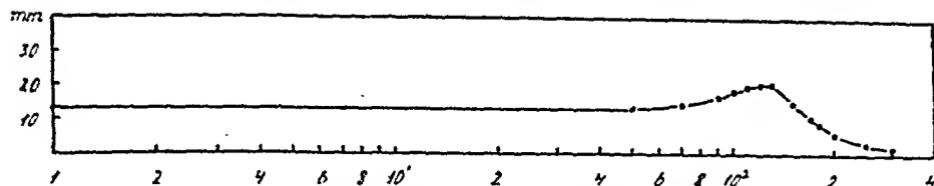


Fig. 8. Dynamic calibration of the pressure recorder.

Abscissa: frequency in cycles per second, log. scale. Ordinate: amplitude in mm.

Hysteresis of the condenser chamber proved to be without significance and maximal bending of the entire cable may produce capacity changes which at most correspond to 5 mm Hg. During the experiment bending or vibration only occur in the thin part of the cable causing no capacity changes whatever. Static and dynamic pressures are recorded with the amplifier and a torsion wire oscillograph of a standard electrocardiograph and care is taken to work in the linear range of this system.

When pressure curves, obtained shortly after the operation when the animal is still affected by the narcosis, are compared with those taken 1—3 days later definite differences are observed. On the first arrhythmia is pronounced with consequent variations of the height of systolic and diastolic pressures. The diastolic pressure in particular can become much reduced during a long pause.

The form of the pulse is likewise altered both regarding the general shape and in certain details. This is due not only to the reduced frequency which draws out the pressure variations, but mainly to the fact that the initial rapid systolic rise as affected by the narcosis, is a much smaller fraction of the total rise than normally, while the subsequent pressure fall before the minimum of the incisura is also much less pronounced. The semilunar valves are closed, therefore, at a higher pressure, relatively, than in the normal animal (Fig. 9). The diastolic part of the curve is changed less, but is often more smooth without the slight initial rise usually seen in normal animals at rest. The secondary pressure vibrations are less distinct or absent. The cause of these vibrations has been an object of intensive discussion in the literature and will be dealt with in the following paper.

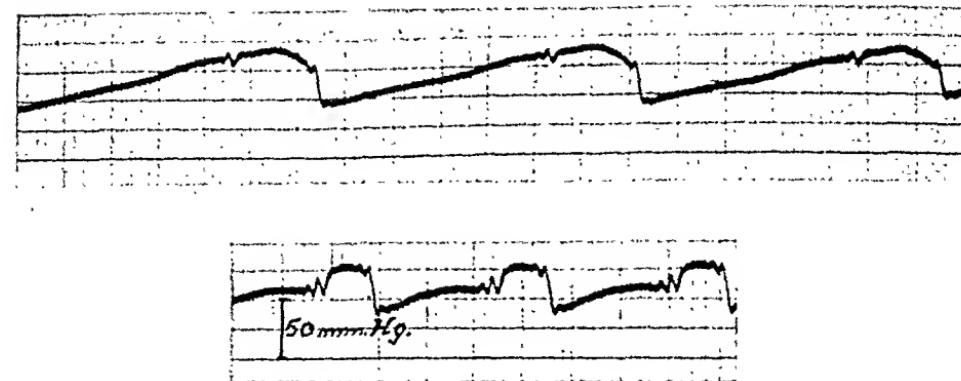


Fig. 9. Pressure curves from the aorta. a) during narcosis. b) 72 hours later.

Abscissa: time marks for 1/10 and 1/50 sec.
Ordinate: mm. (1 mm=5 mm. Hg). The curve has to be read from the right to the left.

To illustrate the application of the method I shall describe briefly two experiments on a dog doing light work on a treadmill. The dog, a young bitch of 11 kg. was trained on the treadmill and perfectly familiar with it before the operation.

Exp. 1.

Rate of mill 3.6 km/hour. No incline of the mill.

	Blood pressure in mm. Hg.		pulse rate
	systolic	diastolic	
Dog at rest before work	135	120	173
Just after start	180	140	173
After 3 minutes	170—175	130—115	150
" 4 "	165—154	130—116	150
Work just finished	156	116	—

The dog was difficult to get started and tried to stop twice during run, being frightened by the manipulations necessary for the pressure registration.

Exp. 2.

Rate 6 km/hour. About 5 degrees incline.

	Blood pressure in mm. Hg.		pulse rate
	systolic	diastolic	
Dog at rest before work	128—125	90—85	156
Just after start	164—170	130—134	155
After 1 minute	162—171	125—128	187
" 2 "	158—180	136—146	190
" 3 "	157—171	113—135	176
" 5 "	151—167	104—122	201

¹ The high pulse rate before work is possibly caused by fright.

Also in this case the dog was difficult to get started, but ran afterwards quite normally, except for a short period about the third minute.

Pressure curves were obtained for the complete transition period from work to rest (Fig. 10). They show quite gradual reduction in pressures and pulse rate from 166/115 mm Hg with a pulse of 214 to 140/100 mm Hg, pulse 176.

It should be noted that the absence of an increased pulse frequency at the beginning of work is at variance with the results obtained in studies on man (KROGH and LINDHARD, 1913) and the pressure changes found are difficult to explain. They may possibly be caused by fright, but the mechanism is obscure.

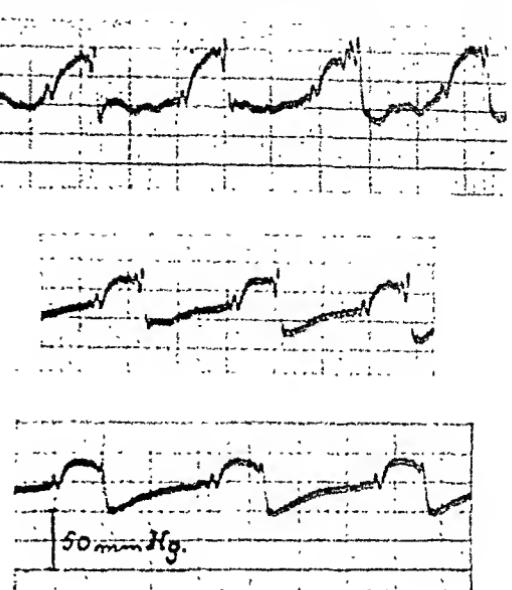


Fig. 10. Pressure curves in the transition period from work to rest.
Abscissa and ordinate: see fig. 9.

a) during work on a treadmill. 5 min. work.
b) transition from work to rest. c) curve from the resting dog (10 sec. between b and c).

The curves are read from the right to the left.

Summary.

An apparatus is described allowing photographic recording of absolute arterial pressures and rapid pressure variations in dogs even during movement.

Aortic pressure curves from normal animals are compared with those obtained shortly after narcosis and operation.

Two experiments are presented in which the pressures were recorded during light muscular work and in the transition from work to rest.

The author wishes to thank F. BUCHTHAL, M. D., for advice and encouragement in the course of this work.

References:

- BUCHTHAL, F., and E. WARBURG, Acta physiol. scand. 1943. 5. 55.
- KROGH, A., and J. LINDHARD, J. Physiol. 1913. 47. 112.
- SCHÜTZ, E., Klin. Wschr. 1937. 16. 1132.
- WETTERER, E., Z. Biol. 1943. 101. 332.

From the Laboratory for the Theory of Gymnastics,
University of Copenhagen.

The Morphology of the Pulse Curve of the Aorta.¹

The Secondary Oscillations.

By

ARNE P. SKOUBY.

Received 17 September 1945.

The secondary oscillations appear just before the rapid systolic rise in pressure in the last phase of this rise and again shortly after the systole and are designated by FRANK as pre-oscillations, initial oscillations and incisura with consequent after-oscillations. (1905). He also gave an interpretation of their single elements which was in the main accepted by later investigators. The pressure curves formerly studied were registered from exposed vessels of anaesthetized animals under artificial respiration and most frequently with the chest opened and therefore in conditions far from normal. Sometimes they were also recorded both in animals and man by an appliance which magnified and recorded changes in diameter or volume of unexposed vessels.

In order to examine the oscillations in more natural circumstances I have by means of the technique described in the preceding paper recorded pressure curves from the root of the aorta in dogs both during narcosis in connection with the insertion of the catheter and 1—3 days later *without any narcosis*. In some cases records were taken at the start of a short muscular work on a treadmill, during work, at the end of it and just after. Furthermore the influence of adrenaline and amylnitrite has been studied.

During narcosis the pre-oscillations and initial oscillations were not distinct and the after-oscillations only just visible. In normal

¹ The expenses of the work have been defrayed by the Frk. P. A. Brandts Legat foundation.

animals, however, pre-initial and after-oscillations are as a rule very distinct so that the distances between maxima and minima can be measured and the corresponding time difference calculated with an accuracy of about 1 millisecond. After their position the maxima and minima are denoted by letters as seen in fig. 1 and in the following table the distances between them are given in milliseconds from curves recorded from three dogs in varying conditions.

The values given for the first dog are averages of 9—14 continuous pulses, but for the second and third animal of 5 continuous pulses only. For the distances b_1c and cd there are considerable differences between the single pulses amounting even to 50 % of the average value, but for the other distances the variations are only 1—2 milliseconds corresponding to the error of measurement.

Table.

The table represents distances between maxima and minima (denoted by letters of fig. 1) of pressure pulso curves from three dogs in varying conditions. The blank spaces of the table are due to the fact that the distances could not be measured from the pulse curves in question.

		aa ₁	a ₁ b	bb ₁	b ₁ e	cd	de	ef	fg	gg ₁	g ₁ g ₂	hi	ij	jk	kl	lm
dog. 11 kg.	Narcosis			9	7.19	7	9					11	9	9		
	Rest before work	9	10	9	8.20	7.11	10	10				10	11	10	10	10
	At the beginning of the work		9	9	3.16	7.10						9	9	8		
	After 5 minutes work				4.14	5.8	6	8	8	8		7	8	8		
	Rest just after work			8	6.19	6.10					9	9	10			
dog. 12 kg.	Rest before adrenaline...	6	5	5	5.24							7	5	5		6
	5'40" after adrenaline...	7	6									8	5	7		
	Rest before work		6	6	12.17	4.10						7	7			
	Rest just after work		5									5				
dog. 22 kg.	Narcosis			8	6.64	16						10	8	9		
	Rest before amylnitrite...	9	7	7	4							9	6	7		6
	After amylnitrite-inhalation. (Very anxious)....			7						fg ₁	12		8	6	7	

It turns out that apart from the intervals b_1c , cd and de all distances measured in the single experiment are practically uniform. From animal to animal and in the same animal under varying conditions the distances between maxima, though constant in the single determination, may have different values. The rise in pressure at the moment of inflow of blood into the aorta shows differences corresponding to the variations of b_1c . Between c and d often one or more notches are present (Fig. 2), the point d being the first pronounced maximum found.

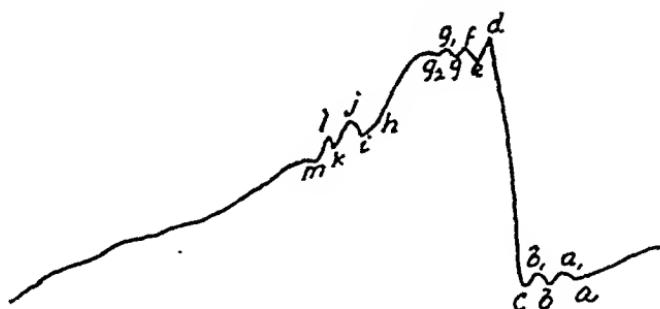


Fig. 1. Schematic pressure pulse curve with the secondary oscillations denoted by letters referred to in the text. The curve is to be read from the right to the left.

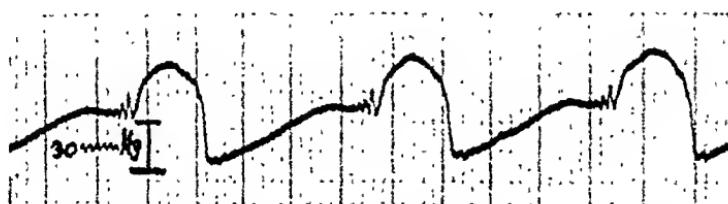


Fig. 2. Pressure pulse curves from the root of the aorta from a dog at rest showing a notch before the first pronounced maximum found. The curve is to be read from the right to the left. Time marks $\frac{1}{50}$ and $\frac{1}{10}$ sec.

Finally it can be seen that the initial oscillations are pronounced when the systolic curve continues as a plateau after the steep rise. The initial and afteroscillations have the character of damped oscillations with gradually decreasing amplitude.

Discussion.

According to FRANK the pre-oscillations, of which one or two are usually observed, are an expression partly of the apex beat cardiogram and partly of the ventricular pressure. The first pre-oscillation is thus taken to be caused by the systole of the atria and the

second by the ventricular systole, an explanation which is accepted by R. and C. TIGERSTEDT (1908, 1918) and also by v. BORN (1911). The aortic valves are taken to move with a certain force towards the aorta.

The initial oscillations FRANK considers in his first papers (1905) as oscillations of the elastic system represented by the heart and central arteries. Later (1925—26) he goes more deeply into the matter and says that they are perhaps a part of the cardiogram, but that they also represent a wave reflected from the carotid artery when blood suddenly enters the aorta. He takes this view to be supported by the fact that their time relation to the opening of the semilunar valves corresponds to twice the length of the carotid.

The after-oscillations are taken as reflected and due to a reperfusion of the blood against the closed semilunar valves. The incisura, the sudden pressure fall at the end of systole is due according to FRANK to a real return flow of blood at the close of ventricular contraction. That it is no oscillation proper is demonstrated according to him by the fact that the period is shorter than that of the shortest possible oscillation from the carotid and that the descending part is sometimes less steep than the ascending in contradistinction to the rule for an oscillation proper. This analysis applies especially at normal or supernormal blood pressure. At low pressure, as produced by inhalation of amyl-nitrite, conditions are different. FRANK writes: "The reduced rate of propagation caused by the low blood pressure brings about a delay in the large waves and especially in the carotid wave which is the first wave to return from the periphery. The period during which the pulse pressure is proportional to the rate and can be used as an expression for it thereby is increased. While at normal blood pressure the carotid wave only shows up as a more rapid rise preceded by a point of inversion a maximum is now formed. The velocity curve extends to the following minimum, then the carotid wave sets in decidedly and causes a new maximum. During the further course of the poisoning, the rapid systolic rise will be exceedingly steep. The maximum of steepness and maximum of pulse rate are reached simultaneously. The first (maximum of velocity (d, fig. 1)) will be at a higher level than that of the carotid wave which finally only can be discriminated in the descending part of the curve. The pressure curve later falls very steeply towards the incisura, which is essentially more prolonged

than normal probably because the oscillation frequency of the valve-tube-fluidsystem is essentially reduced."

A curve resembling that described above by FRANK was published by v. BORN (1911) after stimulation of the vagus and the depressor nerves, but the phenomenon is not discussed.

In his monograph WIGGERS (1928) states, that the secondary oscillations are free oscillations in the elastic structures and the column of blood and make their appearance when a force is suddenly applied or withdrawn.

BRAMWELL and HILL (1925) suggested that such vibrations were produced by a turbulent condition in the blood stream, brought about by the wave front having become unstable.

It follows from the above that it is generally assumed that initial and after-oscillations are oscillations proper. The pre-oscillations are, however, taken to originate from the atria and ventricle systole, which moves the semilunar valves towards the aorta *i. e.* a quite different mechanism.

The present results indicate that both pre-initial- and after-oscillations are oscillations proper of the vascular system. They have the same frequency even in different conditions. *As the pre-oscillations begin before the expulsion period it is probably the pull of the heart on the aorta during the isometric phase of the contraction which initiates the oscillations* and not as FRANK supposes the movement of the aortic valves. This pull sets in with varying rapidity. The ease with which the oscillating system responds may vary according to its stiffness and blood content and even the position of the catheter may be a factor. When its opening is situated close to the arterial wall or to the valves there may be a direct transmission of the oscillations of the vascular wall.

The point c on fig. 1 represents the moment when pressure suddenly begins to rise and this may happen at a maximum or a minimum of the pre-oscillations or any moment in between, explaining the differences of b_1c in the same experiment. In accordance with FRANK the origin of the initial oscillations may be explained by the inflow of blood in the aorta. They start somewhere between c and d and may cause the notches mentioned above. A rapid expulsion of a large blood volume will cause a large amplitude of the oscillations and the sudden transition from a steep rise to a plateau or even a slightly falling pressure will make them further prominent. This I think, explains the differences between curves obtained during rest and those taken at

work and also the fact that FRANK in experiments with amylnitrite finds two maxima, the first rising above the second. In the later the systolic rise in pressure is directly continued by a plateau or a falling pressure.

The point d in fig. 1 corresponds to the moment at which the two interfering curves viz. the fundamental systole and the superimposed curve of secondary oscillations form a visible maximum. The time relation of this point depends upon the rate and duration of the rise in systolic pressure and the frequency of the secondary oscillations.

Regarding the incisura I have not observed the negative wave mentioned by FRANK (1905), but usually seen a gradual slope from the top of the curve to the minimum of the incisura. In some cases there is a definite change in the curve at h (Fig. 1), which may correspond to the closing of the valves. With FRANK I suppose that the after-oscillations are due to the pressure fall in the aorta and the repercussion of the blood against the closed semilunar valves.

Summary.

In order to examine the secondary oscillations in more natural circumstances than done before, I have by means of a condenser manometer recorded pressure curves from the root of the aorta of dogs during narcosis and *without any narcosis in varying circumstances*. The distances between the maxima and minima of the oscillations are measured and the following results found:

- 1) Apart from the intervals b₁c, cd and de (Fig. 1) all distances measured in the single experiment are practically uniform.
- 2) In different animals and in the same animal in varying conditions the distances between maxima, though constant in the single determination, may have different values.
- 3) The pressure curves at the moment of inflow of blood into the aorta shows differences corresponding to the phase variation of the preoscillations.
- 4) The initial oscillations are pronounced when the systolic curve continues as a plateau after the steep rise.
- 5) The initial and after-oscillations have the character of damped oscillations with gradually decreasing amplitude.

The results are discussed and indicate, that the pre-oscillations in opposition to FRANK's theory are oscillations proper of the same

system to which initial and after-oscillations belong. As the pre-oscillations begin before the expulsion period it is supposed, that the pull of the heart on the aorta during the isometric phase of contraction initiates the oscillations.

An explanation of the differences between curves obtained during narcosis or rest and curves from working dogs or dogs during amylnitrite inhalation is forwarded.

The author wishes to thank F. BUCHTHAL, M. D. for advice and encouragement in the course of this investigation.

References.

v. BORN, M., Skand. Arch. Physiol. 1911. 24. 127.
BRAMWELL, J. C., Heart 1925. 12. 23.
FRANK, O., Z. Biol. 1905. 46. 441.
FRANK, O., Sitzungsber. d. Ges. f. Morph. u. Physiol. in München 1926. p. 33. and 1927. p. 32.
TIGERSTEDT, C., Skand. Arch. Physiol. 1918. 36. 101.
TIGERSTEDT, R., Ibidem 1908. 20. 249.
WIGGERS, C. J., The Pressure Pulses in The Cardiovascular System. London 1928.

From the Department of Medical Chemistry, University of Uppsala.

On the Presence in Extracts of Leaves and Plants of a Factor Interfering with the Chemical Determination of Thiamin by the Method of Melnick and Field.

By

GUNNAR ÅGREN.

Received 19 September 1945.

During the investigation of the general occurrence and properties of a thiamin-destroying enzyme (LIECK and ÅGREN 1944, ÅGREN 1945, WIKÉN and ÅGREN 1945), it was found that extracts of leaves from certain trees and plants contained a factor which in some respects behaved as this enzyme. The present paper reports the occurrence of the factor as well as some of its chemical properties.

Experimental.

Preparation of extracts. In the determination of the general occurrence of the factor, the extracts were prepared in the following way. About 50 g. of the fresh material stored at — 15° C. overnight were finely ground by means of a meatmincer. The pulp was shaken for 1 hour at room temperature with the double volume of distilled water and centrifuged for 1 hour at 3000 r. p. m.

Thiamin determinations. Thiamin was determined with some slight modifications of the method previously employed (LIECK and ÅGREN 1944). In the beginning of the investigation 1 ml. aliquots of a thiamin hydrochloride solution containing 2 micromoles (2×10^{-6} mol) of thiamin were incubated with 3 ml. of extract, neutralized when necessary to pH 7.4 and 1 ml. of 0.05 M phosphate buffer pH 7.4 for 2 hours at 40° C. 5 ml. of 20 per cent trichloroacetic acid solution were added. The solution precipitated with trichloroacetic acid was allowed

to stand for 30 minutes in order to obtain complete precipitation. After centrifugation, 2 ml. aliquots of the supernatant fluid were analyzed for thiamin and compared with a solution which, apart from the fact that the incubation had been omitted, was in every respect identical. Subtraction of the number of micrograms thiamin found in the digested sample from that of the control sample gave the amount of thiamin "destroyed" by the factor. This procedure was used in the part of the investigation where the occurrence of the factor was determined. When it was found that the effect of the factor was rather rapid, the incubation procedure was abandoned and henceforth the control samples consisted of 1 ml. of thiamin solution, 3 ml. of distilled water and 1 ml. of buffer. The unit of "activity" was defined as that amount which under the above conditions would cause the "disappearance" of 1 micromol of thiamin (molecular weight of thiamin hydrochloride taken as 337.3). In practice a preliminary assay was made with several dilutions of the unknown extract. The assay was then repeated using the amount calculated to contain about 1 unit of activity.

Results.

I. The occurrence of the factor. The results obtained with extracts from a series of plants and leaves are given in Table 1. The interest was especially focused on material more commonly used for nutritional purposes.

A comparison of the values of the digestion samples with those of the control samples immediately demonstrated the small difference between the figures. The "activity" of the factor seemed to be rather rapidly displayed and the incubation procedure was therefore abandoned. In the preparation of the factor, the leaves of birch, oak and aspen seemed to be the most promising material. A series of determinations showed that extracts of aspen and oak contained about 50—70 units of activity per 3 ml. of extract while the corresponding figure for birch extract was only 10—20 units. As the extracts of birch and oak seemed to contain rather high amounts of mucous substances, the attempt to isolate and study the chemical properties of the factor were carried out with extracts of aspen leaves.

II. The stability of the factor. The factor seemed to be rather thermostable, at least in the less purified solutions. Extracts heated for 3 minutes at 100° C. still contained the main part of the original activity. When kept at room temperature, the extracts did not lose any activity in 2 days. A sample of aspen leaves was dried at room temperature to constant weight and with intervals

Table I.

On the occurrence of a factor interfering with the chemical determination of thiamin in extracts of some Swedish trees and plants.

The values refer to the extinction values given by the diazotized vitamin B₁ content of 2 ml. aliquots of trichloroacetic acid centrifugates from 3 ml extracts incubated for 2 hours with 2 micromols of thiamin in the digestion tests = D. T.
Control tests = C.T.

Species	Latin name	Sample	Extinction value of	
			D. T.	C. T.
Alfalfa	Medicago sativa L.	Whole plant	0.17	0.20
Aspen	Populus tremula L.	Leaves	0	0
Birch	Betula Sp.	"	0	0
Birdcherry	Prunus padus L.	"	0	0
Bird's-foot	Lotus corniculatus L.	Whole plant	0	0
Blueberry	Vaccinium myrtillus L.	Leaves	0	0.02
Brake	Epteris aquilina	"	0	0.02
Buttercup	Ranunculus acris L.	Whole plant	0.02	0.04
Carrot	Daucus carota L.	Tubers	0.10	0.14
Carrot		Leaves	0.16	0.18
Celery	Apium graveolens L.	Whole plant	0.15	0.21
Chive	Allium schoenoprasum L.	Leaves	0.14	0.19
Cucumber	Cucumis sativus L.	Fruit	0.11	0.14
Jerusalem artichoke	Helianthus tuberosus L.	Tubers	0.10	0.14
Lettuce	Lactuca sativa L.	Leaves	0.16	0.19
Lily-of-the-valley	Convallaria majalis L.	"	0.01	0.05
Marsh marigold..	Caltha palustris L.	Whole plant	0.13	0.13
Oak	Quercus robur L.	Leaves	0	0
Onion	Allium Cepo L.	Tubers	0.17	0.16
Onion		Leaves	0.12	0.17
Parsley	Petroselinum sativum Hoffm.	"	0.12	0.14
Potato	Solanum tuberosum L.	Tubers	0.13	0.17
Red clover	Trifolium pratense L.	Whole plant	0.13	0.19
Rhubarb	Rheum undulatum L.	Stalk	0.11	0.18
Spinach	Spinacia oleracea L.	Leaves	0.17	0.24
Summer-radish ..	Raphanus sativus L. f. radicula	Tubers	0.24	0.23
Summer-radish ..		Leaves	0.17	0.16
Swedish turnip ..	Brassica Napobrassica Mill. f. esculenta	Tuber	0.09	0.15
Timothy	Phleum pratense L.	Whole plant	0.10	0.15
Wild cerefolium ..	Anthriscus silvestris Hoffm.	Whole plant	0.16	0.19

of two weeks the activities of freshly made extracts were determined. Each time 10 g. of ground leaves were extracted with 40 ml. of distilled water. The activity remained constant during more than two months. Extracts were also made on withered leaves of aspen and brakes from the previous year. They still contained 0.5 and 2 units per 3 ml. of extract respectively.

The pH-stability of the factor was determined at pH 1.6, 4.0, 7.4 and 10.0. To different dilutions of an extract were added the necessary amounts of hydrochloric acid or sodium hydroxide to bring the hydrogen ion concentration to the appropriate values, whereafter equal volume of the different buffer solutions were added. The activities were determined after an incubation period of 24 hours at room temperature on the neutralized solutions. The results of a typical series of experiments are given in Table 2.

Table 2.

The pH-stability of the factor in extracts of aspen leaves.

The figures refer to the extinction values given by the diazotized thiamin content of 2 ml. aliquots of trichloroacetic acid centrifugates from different dilutions of a concentrated dialysate of extract of aspen leaves.

Buffer	Extinction value of		
	Extract diluted 1 : 200	Extract diluted 1 : 500	Extract diluted 1 : 1000
Glycine-HCl buffer pH 1.6 (SÖRENSEN)	0	0.07	0.15
M/10 acetate, pH 4.0 ..	0	0.07	0.14
M/15 phosphate, pH 7.4	0	0.08	0.16
Borate-NaOH buffer pH 10.0 (SÖRENSEN)	0.04	0.14	0.21
Glycine-NaOH buffer pH 10.1 (SÖRENSEN)	0.06	0.15	0.23

It is obvious that the factor is more stable in acid and neutral solutions than in alkaline solutions. The activity of the factor was rather resistant against treatment with 5 and 10 per cent solutions of hydrogen peroxide. These experiments were carried out with the technique previously used in similar investigations of the stability of the thiamin-destroying fish enzyme. (ÅGREN 1945).

III. The purification of the factor. Preliminary experiments demonstrated that the factor was rather soluble in ethyl alcohol but only inconsiderable amounts of activity could be directly extracted with ether from neutral or slightly acid solutions. In fact the ethyl ether used in this laboratory (Swed. Pharmac. Ed. X) contained small amounts of substances which interfered with the chemical determinations of the vitamin. The leave-factor dialyzed through cellophane membranes. The following method of preparation was finally used:

1. About 2 kg. of frozen leaves were finely ground and shaken for 1 hour at room temperature with the double volume of distilled water and centrifuged for 1 hour at 3000 r. p. m. The centrifugate was dia-

lyzed in 7 cellophane tubes, $\varnothing = 40$ mm., at 6° C. for 24 hours with constant stirring against distilled water which was renewed after 8 and 18 hours. The content of each cellophane tube was in this way dialyzed against 5000 ml. of water. The contents of the cellophane tubes, which still contained about 20 per cent of the original activity, were discarded. Direct determinations of the activity of the dialysable portions obtained after 8, 16 and 24 hours of dialysis gave the following values: 2 units, 1 unit and 0.5 units per 3 ml. of dialysate. Thus the main part of the activity was dialyzed in 16 hours or less.

2. The dialysable portions were concentrated *in vacuo* to 250 ml. and absolute ethyl alcohol was added to a final concentration of 90 per cent. The precipitate, which contained about 5 per cent of activity, was washed with 90 per cent alcohol and then discarded. The collected alcohol solutions were concentrated *in vacuo* to about 50 ml. and precipitated with absolute alcohol to a final concentration of 90 per cent. The precipitate, which contained about 1 per cent of the activity of the sample, was washed with a little 90 per cent alcohol and then discarded. To the centrifugate were added two volumes of ether and a fine, inactive precipitate which formed was centrifuged off. The alcohol-ether centrifugate was concentrated *in vacuo* to 50 ml. after two additions of 100 ml. of distilled water. During the concentration inactive crystalline precipitates sometimes appeared. These were centrifuged off. The centrifuged solution was stored at -15° C. After 24 hours a heavy, white and inactive precipitate had been formed. After centrifugation the remaining brown-coloured solution contained about 30,000 units, or about 30 per cent of the activity of the original extract. The purified solution contained 250–300 mg. of dry material per ml., which means that 0.8 mg of substance contained the amount of activity which caused the disappearance of 1 micromol of thiamin when determined by the MELNICK and FIELD reaction.

3. A further purification was achieved by means of electrodialysis. The apparatus described by THEORELL and ÅKESSON (1942) was used. 10 ml. portions of the purified solution from 2 was used in a dilution of 1 : 5. With these amounts of material the experiment was completed in about 4 hours. The strength of the current was never permitted to exceed 60 milliamperes. Analysis of the contents of the three compartments at the end of the experiment demonstrated that the pH was 7.1 in the cathode compartment, 6.0 in the middle compartment and 3.0 in the anode compartment. Of the original 700 units about 50 units had migrated into each of the anode and cathode compartments. The remaining 600 units were found in the middle compartment. The results were easily reproducible. The solution from the middle compartment contained 30 mg. of dry material per ml. Calculated per unit weight, about half of the material had migrated into the anode and cathode compartments. About 0.5 mg. of dry material from the solution of the middle compartment caused the disappearance of 1 micromole (0.3 mg.) of thiamin.

Subsequently the electrodialysis was carried out with samples of about 2 g. of dry material in 15 ml. of solution and the experiments

were run overnight. The contents of the middle compartment were evaporated to dryness and again extracted with absolute ethyl alcohol, where some inactive crystalline material was insoluble. The alcohol solution was evaporated to dryness and a yellow-coloured material obtained. Through repeated extractions with small amounts of alcohol some inactive material could be removed. In this way preparations were obtained from which about 0.3 mg. of material caused the disappearance of 1 micromol of thiamin. The yield amounted to about 20 per cent of the activity in the original extract.

Analysis. The most active preparations seemed to be free from nitrogen as determined by the micro-Kjeldahl procedure.

phosphorus as determined by TEORELL

sulphur as determined by GROTE-KREKELER.

Each analysis was carried out with about 100 mg. of dry material.

For the analysis of carbon and hydrogen the substance was dried at 100° C. *in vacuo* over P₂O₅. Found C 51.3 %, H 6.44 %. The substance was free from ash.

Solutions of the purified factor reduced Fehling's solution but not Tillman's reagent. The following phenol-reactions were carried out:

FeCl₃-reaction 1: 100 water solution = green, after the addition of sodium acetate = violet colour.

Millon reaction 1: 1000 water solution = yellow colour

Liebermann reaction 1: 1000 water solution = green colour

Guareschi-Lustgarten reaction 1: 100 water solution = no colour

Pougnat reaction 1: 100 water solution = no colour.

Discussion. In this paper a factor is described which causes the disappearance of thiamin as determined by the method of MELNICK and FIELD. The factor is not present in any considerable amounts in the commonly used vegetables or in cattle feed as timothy, clover or alfalfa. On the other hand, fresh and dried leaves of aspen and birch, which in this country are used as cattle and sheep fodder, contain high amounts of the factor (see Table I). This fact is of interest with regard to the general application of the method of MELNICK and FIELD. From the chemical investigations it is obvious that the factor has a low molecular weight, is easily soluble in absolute alcohol and behaves as an electro-neutral substance at electrodialysis. The factor seems to be free from phosphorus, nitrogen and sulphur. The figures obtained in the elementary analysis as well as the colour reactions given by the purified material are in agreement with the presence of substances of phenolic character. In accordance with these results it

was found that small amounts of the factor were present in the condensate obtained at the concentration *in vacuo* of the dialysate. A closer investigation of the chemical properties and attempts at further purification of the factor are under way.

The mode of action of the factor is not clear. Either such a factor may interfere with the coupling of the reagent and the quaternary nitrogen in the thiazole nucleus of thiamin or the activity of the factor may be associated with a direct change of the thiamin molecule. The first possibility has previously been investigated by PREBLUDA and MC COLLUM (1939). According to these authors, the following nitrogen-, phosphorus- and sulphur-free substances should *not* interfere with the chemical determination of the vitamin: acetone, dextrose, ethyl alcohol, fructose, galactose, hydroquinone, inositol, lactose, methyl alcohol, phloroglucinol, resorcinol, sucrose, theelin. Some of these results have been checked by the present author. If 2 micromoles of thiamin are to be determined in the presence of about 100 mg. of any of the above mentioned monosaccharides, only 1 micromol of thiamin can be discovered by the chemical method. The same effect can be obtained with as little as 0.3 mg. of the present factor. The second possibility, a direct change of the structure of the thiamin molecule, is at present being investigated by microbiological methods. This alternative is possibly favoured by the reducing property of the factor, which may destroy the thiamin molecule in a way similar to that of sodium hyposulphite, cysteine or glutathione (compare ÅGREN 1945).

Summary.

A factor which interferes with the chemical determination of thiamin as carried out by the method of MELNICK and FIELD has been isolated from extracts of aspen leaves. The factor is an electroneutral, low-molecular substance, probably with reducing properties and not containing sulphur, phosphorus or nitrogen. The general occurrence of the factor in leaves from plants and trees and in vegetables has been investigated.

The writer is indebted to the Ferrosan Corporation for a grant which supported the present investigation. He further acknowledges his thanks to Miss Ahlquist and Mr. Eklund for valuable assistance throughout the investigation.

References.

LIECK, H., and G. ÅGREN, Acta Physiol. Scand., 1944. 8. 203.
MELNICK, D., and H. FIELD, J. Biol. Chem. 1939. 127. 515.
PREBLUDA, H., and E. MC COLLUM, Ibidem 1939. 127. 495.
TEORELL, T., Biochem. Z. 1931. 230. 1.
THEORELL, H., and Å. ÅKESSON, Ark. Kemi, Mineral. Geol. 1942.
16A. No. 8.
WIKÉN, T., and G. ÅGREN, Ark. Botanik 1945. 32. No. 9.
ÅGREN, G., Acta Physiol. Scand. 1945. 9. 221.

ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 10. SUPPLEMENTUM XXIX.

*From the Medico-physiological Laboratory, Medical Clinic I,
Karolinska Sjukhuset, Stockholm*

ON THE CONCENTRATIONS OF SOME
SULFANILAMIDE DERIVATIVES
IN DIFFERENT ORGANS AND
TISSUE STRUCTURES

*A histo-pharmacological study by means of fluorescence
microscopy with some aspects on the possibilities of obtaining
high concentrations of chemo-therapeutics in certain tissues*

by

STURE HELANDER

STOCKHOLM 1945

Translated by

ULLA SCHÖTT

Contents

	page
Preface	5
Introduction	7

Part I. Methods.

Chapter I. Prerequisites of histo-pharmacological investigations	11
Fixation method	11
Detection method	14
Chapter II. Regarding the possibility of detecting drugs by means of their fluorescence	17
Definitions	17
Chemical constitution and fluorescence	18
Physical factors affecting the fluorescence	20
The fluorescence of various drugs	21
Chapter III. The detection of drugs in tissue sections by means of fluorescence microscopy	23
Previous investigations	23
Own investigations	27
The fluorescence of heated drugs	27
The fluorescence of tissues after heating	30
The detection of drugs in tissues after heating the sections	32

Part II. Problems and results.

Chapter IV. Aspects of the problems and earlier investigations regard- ing the relationship between the effect and localization in the tissues of sulfanilamide derivatives	39
The therapeutical effect of sulfanilamide as compared to prontosil rubrum and soluble	39
Animal experiments	39
Clinical examinations	42
Earlier investigations regarding the distribution of sulfanilamide derivatives in the tissues	46

	page
Chapter V. Own investigations regarding the localization of certain sulfanilamide derivatives to different tissue structures in various organs by means of fluorescence microscopy	53
Material	53
The distribution of prontosil soluble in the tissues at different periods after intravenous injection	54
The distribution of prontosil soluble in the tissues 12—24 hours after repeated doses	61
The distribution of prontosil soluble in the skin after local application	61
The distribution of sulfanilamide and sulfathiazole in the tissues at different periods after intravenous injection	62
Survey of the results	64
Summary	66
Chapter VI. Investigations regarding the localization and passage of sulfanilamide derivatives to the central nervous system	68
Introduction	68
Own investigations	69
Discussion of the results	71
Summary	77
Chapter VII. Own investigations regarding the distribution of prontosil soluble and sulfanilamide in different organs, examined by means of chemical methods	79
Introduction	79
Methods and results	79
Summary	83
Chapter VIII. Own investigations regarding the possibilities of directing drugs to certain tissues	84
Investigations regarding the prerequisites for directing sulfanilamide derivatives to elastin and collagen	84
Do any other theoretical possibilities exist of directing drugs to certain tissues?	89
Summary	90
Chapter IX. General discussion	92
Survey of the results	96
Part I	96
Part II	96
References	99

PREFACE

This investigation has been carried out mainly at the Medico-Physiological Laboratory of the Medical Clinic I of Karolinska Sjukhuset in Stockholm.

To Professor NANNA SVARTZ, head of the Medical Clinic, I owe a deep gratitude for the kind interest she has taken in my work, her inestimable advice, and many facilities extended to me.

I am greatly indebted to Dr T. SJÖSTRAND, laborator at the Medico-Physiological Laboratory, who first drew my attention to the possibilities of the fluorescence microscope for the purpose of analysing histo-pharmacological problems, as well as for his never-failing assistance and advice in the course of my inquiries.

My warm thanks are due to Professor T. CASPERSSON for affording me opportunities to discuss various questions in this connection.

I am very much obliged to Dr B. JOSEPHSON, laborator at the Medico-Chemical Laboratory of the Medical Clinic I, for his invaluable help in connection with the chemical part of the work, and also to Dr F. SJÖSTRAND for many useful suggestions and always ready assistance with regard to, inter alia, the histological study of the subject.

I have profited very much from my collaboration with AB Astra and, in particular, I wish to mention Mr BERTIL SJÖGREN, chief chemist, who has in various ways shown generous interest in the progress of the work and whose experiences especially with regard to chemo-therapeutic problems have been of considerable value. Further, Mr T. EKSTRAND and Mr R. DAHLBOM, civil-engineers of the firm, have contributed in producing some of the chemical compounds.

I have much pleasure in acknowledging my indebtedness to my old friend Mr HANS RÅDSTRÖM, Fil. mag., for technical advice and helpful suggestions; to Dr B. OLHAGEN for performing the electrophoresis experiments; to Dr R. FRISK for access to literature; to Dr H. HYDÉN for valuable help in various connections; and to Mrs M. SJÖSTRAND, Miss E. WENCK and Miss B. DAHLBERG for technical assistance.

Finally, I wish to thank my wife for the share she has taken in the work from beginning to end and also for her cooperation in technical details.

The expenses of this investigation have been partially defrayed by the Therese and Johan Andersson Foundation.

The British Council has very kindly contributed to the costs of the publication in English.

Stockholm in April 1945.

STURE HELANDER

INTRODUCTION

PHARMACOLOGY has been defined by CLARK (1937), and others, as the study of the manner in which the functions of living organisms can be modified by chemical substances. For these studies the pharmacologists have long employed physiological and chemical methods.

However, the point of importance is not only the total quantities of a drug to be found in the various organs in their entirety, but also the exact localization in the different tissues. When, for instance, the relationship between dose and effect of drugs is to be examined, the study of the total quantities only, in the tissues, may prove directly misleading. Thus, a substance which acts on different organs and simultaneously circulates in the blood in a certain concentration may be ascertained in a larger total quantity in an organ where it is excreted as, e. g., in the liver and in the kidney, than in the organ on which it exerts most of its action.

Accordingly, an investigation by means of histological methods should be of interest, regarding the localization and concentration of administered drugs to the cells, cellular parts, tissues and tissue structures of the organism, as well as of the factors influencing the distribution of these drugs and the structural changes produced thereby. This type of investigation might be called histo-pharmacological.

Such pharmacological studies are few, largely because of the lack of suitable procedures for fixation and sufficiently sensitive methods for the detection of drugs. Accordingly, in the present paper, Part I will deal with the demands that must be stipulated with regard to the histo-pharmacological methods. A detailed account will also be given of the methods employed, viz. ALTMANN'S freezing-drying method for fixation, and fluorescence microscopy combined, in some instances, with a heating procedure for the ascertainment of the drugs.

As yet, no definite reason has been given for the stronger effect in certain infections, such as erysipelas, obtained by the use of sulfanilamide in an azo linkage than by the equimolecular dose of pure sulfanilamide. Accordingly, an investigation of possible differences in the localization of these drugs in the various tissues was found to be desirable, having in mind also the decisive significance of the concentration at the site of the infection with regard to the chemo-therapeutic effect.

Such differences were established. The particular properties of the substances causing them were submitted to examination. The above-mentioned problems are discussed in Part II where, in addition, the subject of the blood-brain barrier and the transmission of chemo-therapeutics to the central nervous system are dealt with.

PART I
METHODS

CHAPTER I

Prerequisites of histo-pharmacological investigations

Fixation method.

As regards histo-pharmacological examinations of a fixed material, the first prerequisite is a fixation method meeting with the following demands [on the whole, in accordance with F. SJÖSTRAND (1944)]. a) Agonal and postmortal histological changes must not be allowed to occur, b) the fixation should be sufficiently good from a histological (and cytological) point of view to permit careful localization, c) the fixation process must not cause washing out of the drugs or a change in their localization and concentration, d) extraneous substances must not be introduced into the tissues by the fixation and, lastly, e) it must be possible to make thin sections.

As a rule, the usual histological fixation methods, based on fixation in fluids, are unsuitable, the majority of drugs being easily soluble and diffusible. Nor are the usual frozen sections applicable in cases where substances easily soluble in water are concerned, since they may be subjected to diffusion during the melting process. Moreover, it is hard to obtain thin histological sections by means of this method. However, ALTMANN'S freezing-drying technique answers to all the stipulated demands, having been employed in histo-physiological studies by MANN (1902), GERSH (1934), F. and T. SJÖSTRAND (1938), GERSH and CASPERSSON (1940), and F. SJÖSTRAND (1944). (As regards the history, reference may be made to the last-mentioned work.) This fixation procedure is performed in the following manner: Specimens are excised as soon as possible after the animal has been killed. They should

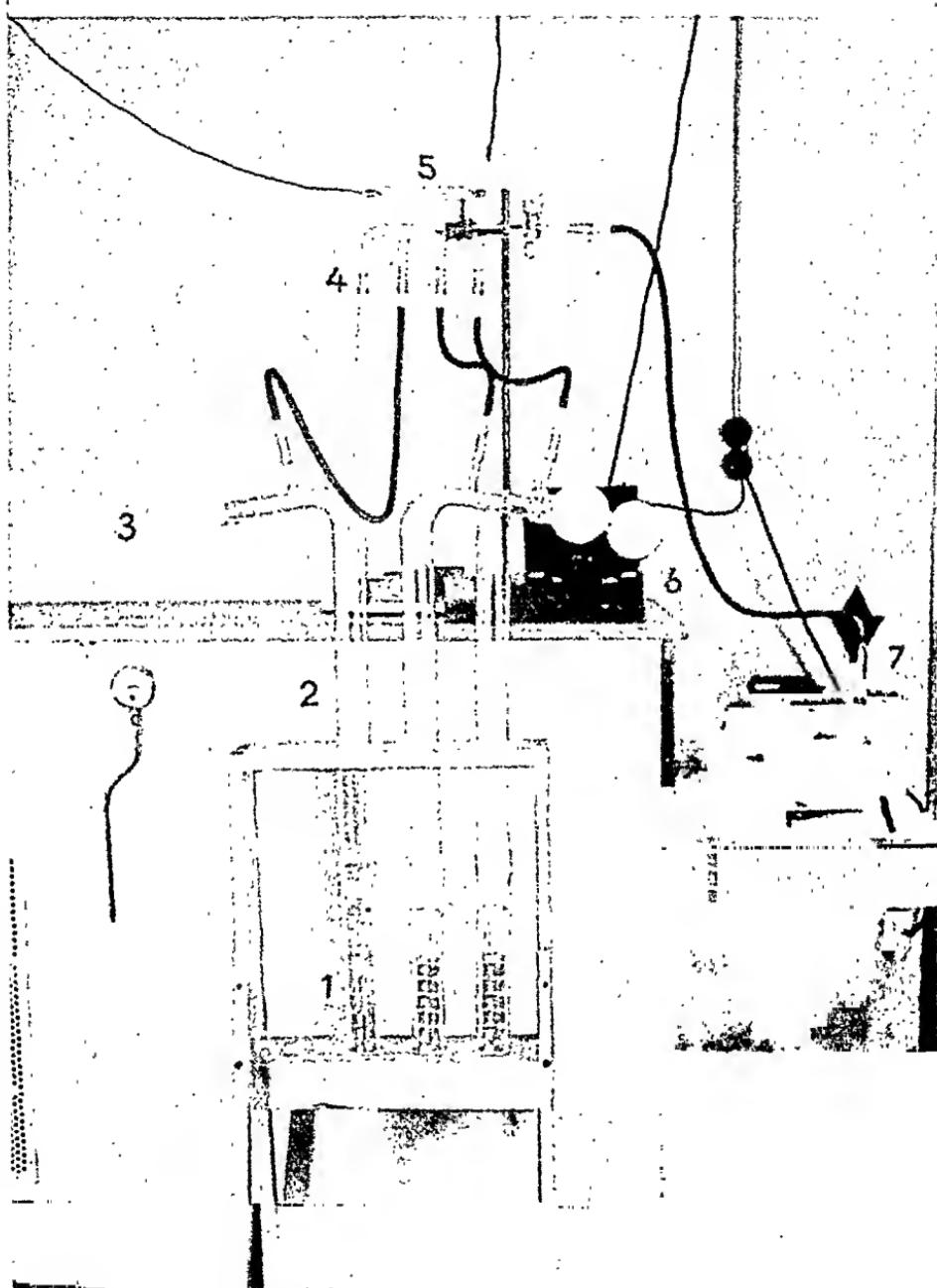


Fig. 1. *The apparatus for dehydration of specimens at $-40^{\circ} C$.
The figures refer to the text on page 13.*

not be too big (approximately 3.10.10 mm is adequate). After rapid freezing in liquid air (-190° C) or in isopentane cooled by liquid air (-170° C , requiring smaller pieces), they are fixed by means of drying in a vacuum over phosphorous pentoxide at -40° C . When dry, the specimens are embedded in paraffin (52° C) and are then ready for sectioning. The thickness of the sections which has generally been used is $5\text{ }\mu$. The slices are placed directly on the slide, without first being extended on water. Approximately one hour in a thermostat at a temperature of $+40^{\circ}\text{ C}$ will generally suffice to smooth out the sections. Proteid glycerine, or some other kind of binding substance for adhesion to the slide, has usually been dispensed with. No special slides have been used.

The apparatus employed (Fig. 1) corresponds, on the whole, to the one described in detail by F. SJÖSTRAND. Only a few minor details have been changed in accordance with the experiences gained by the use of it. The specimens are distinguished by being placed in small enumerated glass receptacles (10 mm in diameter). These vessels are in turn put into metal baskets with perforated sides and bottoms (30 mm in diameter). Each basket holds 5 receptacles. While the glass receptacles in a basket are being filled with specimens, the basket is contained in a Dewar's vessel with solid carbon dioxide (dry ice, -80° C).

When a basket has been filled with specimens, it is deposited in a glass tube (1) which has been placed in the refrigerator beforehand. In order to prevent the basket from turning over, a metal stand, suitably adapted to the size of the basket, has been inserted into the glass tube. The tubes can be filled with 8—10 baskets. There is space in the refrigerator for up to 4 tubes. This has been constructed by AB Elektrolux and will keep a temperature of -40° C . When all the tubes are filled (1), an upper tube (2) is inserted into the lower one, the retorts with phosphorous pentoxide (3) are applied and the various tubes are united in a central one (4). This is then attached to a vacuum pump (7) (Pfeiffer oil rotation pump. Model No. 1810). The vacuum is measured by means of a discharge tube (5) with a high frequency apparatus (6) acting as the current source. The specimens are usually ready when only a faintly blue discharge is visible and the phosphorous pentoxide has ceased to change. This process takes, as a rule, approximately one week, using the size of specimens stated above.

No diffusion has taken place in specimens treated by this method. This is, inter alia, proved by the fact that even exceed-

ingly easily diffusible dyes in high concentrations do not exceed the cellular limit with which they are in immediate contact, whereas, in comparisons with specimens fixed by formalin or alcohol, or with ordinary frozen sections, this result will not be gained. On the other hand, in the latter case, if any dye at all remains, the picture will correspond to the one obtained when a piece of tissue, which has been excised from an animal, is bathed for a few minutes in the same dye solution. A diffuse staining of the specimens will then be seen, irrespective of the cellular limits.

Owing to this method no postmortal histological changes will, evidently, have time to develop. This is, in fact, hardly to be expected since it may be presumed that the majority of the cells are alive during the few minutes required for the excision of the specimens. The freezing itself takes place in the course of maximum a few seconds.

Thus, the Altmann freezing-drying method represents, in general, a useful fixation procedure for histo-pharmacological (as well as histo-chemical, etc.,) purposes.

Detection method.

The second prerequisite, with regard to histo-pharmacological examinations on a fixed material, is that methods for the detection of drugs in sufficiently small quantities are at disposal. Further, these methods should be applicable also to specimens fixed by the aid of methods fulfilling the fore-mentioned demands, without changing the localization or concentration of the drugs on account of the particular method used for their ascertainment. The determining factor with regard to the usefulness of a certain method for histo-pharmacological purposes lies in the concentration limit, i. e. the lowest concentration in which a certain substance may still be demonstrable.

What concentration limit should be determinable by means of a special method, in order to be of histo-pharmacological

value? If a drug is administered at a dosage of 0.01 g/kg of body weight, and this is assumed to be diffusely scattered over the whole body, each cell will then have a concentration of 1: 100 000. If, instead, a drug is dosed at one hundredth mg/kg of body weight, the corresponding concentration will be 1: 100 millions. Accordingly, this would represent the degree of sensitivity to which an ideal histo-pharmacological method must necessarily answer. However, it must hardly be taken for granted that drugs are generally diffusely scattered over the whole body. The fact is that in some organs as, for instance, the kidneys, the liver, and other excretory organs, the concentrations are often considerably higher while in others, e. g., the skeletal system, they are very much lower. Moreover, many drugs are known not to appear within the central nervous system (prontosil soluble, for instance,) others, again, being notable there at comparatively high concentrations. Furthermore, substances with a more specific effect are generally assumed to occur at high concentrations in the parts where their action is exerted, for instance, the glycosides acting on the heart. Nevertheless, as already mentioned introductory, knowledge regarding this matter is, as a rule, very fragmentary.

Is it, on the other hand, at all conceivable that a substance can produce an effect, for instance, on a cell at as low a concentration as 1: 100 millions? According to CLARK (1933), a concentration of 2: 100 millions of acetylcholine is enough to produce a 50 per cent inhibition in a frog's heart. However, the number of molecules per cell is not as low. Thus, a concentration of 1: 1 000 millions of acetylcholine in the frog's ventricular muscle implies about 10 000 molecules per cell.

Even though a method of ideal sensitivity has, as yet, not been discovered, one less sensitive rendering, for instance, concentrations of 1: 1 000—1: 100 000 detectable will, all the same, suffice for the analysis of many problems.

It has long been known that fluorescent substances are observable in very low concentrations owing to this particular property of theirs. Thus, HAITINGER (1937, 1938) states that

the concentration limit of salicylic acid in water is $1: 10^9$, the corresponding values for eosine being $1.33: 10^{11}$, for fluorescein $1.75: 10^{12}$, and for Rhodamin B $1: 10^{12}$, ($1: 1$ million millions).

Some experiments have, in fact, been performed earlier with a view to examine the possibility of following drugs in the organism by means of fluorescence microscopy. The mostly unsuccessful results are probably due, partly, to the unsatisfactory fixation methods and, partly, to the blue fluorescence of the majority of drugs which makes them invisible in the organism, owing to the strong blue auto-fluorescence of the tissue itself which surpasses that of the injected substances.

Even though drugs in water solution have a low concentration limit, the situation is radically changed when drugs are mixed with or absorbed by strongly fluorescent substances, as in the tissues of the organism. However, the matter is simpler in the case of drugs with a fluorescent colour other than blue. Thus, prontosil and other azo-dyes with, for instance, fluorescent colours in red, yellow, etc., were found to be fairly easily detectable even when mixed with other fluorescent substances (HELANDER 1944). The concentration limit of prontosil soluble was determined by dissolving prontosil in blood serum at concentrations down to $1: 100\,000$, whereupon drops of serum were frozen and sectioned in the usual manner. Even at a concentration of $1: 10\,000$, prontosil was still observable in $5\ \mu$ -sections, in spite of the strong blue fluorescence of serum which seems equal in strength to that of the tissue, at any rate in a subjective estimation.

As regards sulfanilamide derivatives with blue fluorescence, a method was elaborated for producing a contrast in colour between the fluorescence of the tissues and that of the drugs. An account of this method will be given in Chapter III.

As the blood concentrations of the sulfanilamide derivatives used for therapeutical purposes lie at $1: 10\,000$ — $1: 20\,000$, it seemed possible that these drugs could be detected by means of fluorescence microscopy in sufficiently low concentrations to render a study regarding their localization in the tissues of interest from a histo-pharmacological point of view.

CHAPTER II

Regarding the possibility of detecting drugs by means of their fluorescence

Definitions

The term photo-luminescence indicates the property of a substance to emit light of a certain wave-length at illumination by rays of another wave-length. There are two different types of photo-luminescence, viz., phosphorescence and fluorescence. They are distinguished merely by a difference in degree, depending on the durability of the luminescence. The term phosphorescence is used when the duration exceeds 0.5×10^{-8} seconds, fluorescence indicating shorter time. A connection occurs between the wave-length of the exciting light and that of the emitted light, defined in Stoke's law as follows: The wave-length of the main part of the emitted light is, as a rule, longer than that of the exciting light. The light absorption of a substance and its fluorescence have certain definite relations, clearly manifested in the connection between absorption spectra and fluorescence spectra. In F. SJÖSTRAND'S paper (pages 11—13), a brief summary dealing with the subject of modern views on the nature of fluorescence can be found.

In principle, rays of any wave-length are capable of producing fluorescence. However, Röntgen rays and ultra-violet rays are those most frequently employed. In fluorescence microscopy, the ultra-violet light has mostly come into use and, generally, the so-called Wood's light. In the latter case, the visible light is removed by filters, and rays of the wave-lengths between 3 000 and 4 000 Å. are mainly used. As a rule, filters absorbing all visible light except a small part of the violet

rays, and with a maximum permeability of 3 500 Å., are employed. When an Hg-lamp serves as a source of light, rays of the wave-length 3 650—3 660 Å. are chiefly used to cause fluorescence.

Chemical constitution and fluorescence.

The question of the relationship between fluorescence and chemical constitution is still far from elucidated, our knowledge being very fragmentary in this respect.

There are extremely few inorganic substances with distinct fluorescence at illumination with ultra-violet rays. When pulverized, a strong fluorescence is stated to emanate from, above all, uranium salts, but also from barium-sulphide, caesium-sulphate, cadmium-wolframate, copper-I-chloride, phosphorous-pentoxide, magnesium-phosphate, zinc oxide, zinc sulphide, mercury chloride ($HgCl$), mercury nitrate, strontium sulphide and thorium chloride (HAITINGER, FEIGL, SIMON 1932).

In comparison, the number of organic compounds with fluorescence is much greater. This applies to the aromatic ones in particular. The majority of the fluorescent substances are derived from benzole and its higher homologues, as well as from many heterocyclic compounds. These groups are called fluorofores, e. g., pyron, azin, oxazin, thiazole, and the thiazin rings, as well as the rings occurring in anthracene and acridin. The fluorescence of a fluorofore group can be intensified by substitution with so-called auxoflore groups, and diminished by diminuflores. Other groups (so-called bathoflores) may cause a deviation of the fluorescence towards a longer wave-length, for instance, from the ultra-violet light into the visible field. This may be illustrated by benzole which gives rise to substances with fluorescence in the visible field by means of substituting it with bathoflore groups. Hypsoflore is the name given to groups causing a deviation of the fluorescence in the opposite direction.

Amino- and hydroxylic groups are auxoflores, as well as their alkyl substitution derivatives, viz. NHCH_3 , $\text{N}(\text{CH}_3)_2$, OCH_3 , and others, which are also bathoflores. Unsaturated carbonic hydrogen residues, such as CH:CH_2 , and cyanic groups, C:N , have the same effect. Carboxylic groups are diminuflore and nitro groups, such as NO , NO_2 , may frequently destroy the fluorescence. Nevertheless, there are exceptions. Thus, m-nitrodimethylaniline, for instance, has a marked fluorescence. Among other fluorescent substances, those containing the azo-group N:N and the azometin-group C:N in a cyclic ring may be noted. However, the simplest compound of this type, viz. pyridin, has no fluorescence.

The saturated groups, such as the alkyls, intensify the fluorescence. The halogens cause only a very slight deviation in the fluorescence but lessen the intensity. This effect increases with the atom weight from fluor to iodine.

The amino groups in the naphthalene and anthracene series are, as a rule, sufficient for producing fluorescence.

The aliphatic compounds generally do not fluoresce as strongly as the aromatic ones, whether in solid or in liquid form (FRANKLIN, ALLEN 1940). Nevertheless, many ketones fluoresce as, for instance, acetone, its homologues and diketones.

Acetylation is stated to have a weakening effect. Salt formation may involve a strong increase in the fluorescence, as for instance in fluorescein, which is explained by the re-arrangement of the molecules, e. g., a quinoid grouping of the atoms. On the other hand, the fluorescence of the aromatic amines is dulled when combined with salts. However, the exceptions from the above-mentioned rules are several. Thus, it may be of a certain value to point out that the fluorescence of pulverized acetyl sulfathiazole is considerably stronger than that of sulfathiazole. Further, HAITINGER states that the azo-dyes have a faint fluorescence. On the other hand, in my own investigations, they have been found to have a very good fluorescence when in a solid form. Apparently, it is as yet impossible

to predict, with any certainty, the degree of fluorescence of a substance, when basing conclusions merely on a knowledge of its chemical constitution.

Physical factors affecting the fluorescence

Owing to the fact that the physical factors influencing the fluorescence of substances have recently been dealt with in a survey account by F. SJÖSTRAND (1944), only a few main points will be briefly mentioned here. Among these factors, attention is first drawn to the *aggregation condition*. The fluorescence of a substance, whether in crystal, powder, liquid, or gas form, may vary not only in intensity, but also in colour and with regard to the fluorescence spectrum. Substances fluorescing in a solid condition have even been found not to do so when in a solution, and vice versa. FRANKLIN and ALLEN (1940) state that many inorganic salts only fluoresce when in a solid form. On the other hand, the fluorescence of many organic compounds may increase in proportion to a reduction of the *concentration*, but becomes weaker again after having attained a certain limit. Relatively speaking, the fluorescence of a substance in solution is more dependent on the surroundings than that of a substance in solid form. The *solvent* plays an important part. It may change the fluorescence colour of the substance as well as its intensity.

Hydrogen ion concentration acts in a similar way, and many fluorescent amphotytes, with the fluorescent colour varying according to the degree of dissociation, may by this means be used as pH-indicators. Solid substances, which do not fluoresce or have but an inadequate fluorescence capacity, may be brought to increase their fluorescence by being *adsorbed* to an adsorbens. They may hereby even be caused to phosphoresce. However, this will not change the fluorescence spectrum.

At high *temperatures* the fluorescence may disappear completely, while at lower temperatures from 0° down to that of liquid air, the fluorescence intensity may increase or only then become visible at all. As will be seen further on, DUTT'S (1930)

observations are of interest in this connection, viz. that many organic substances which have lost their fluorescence after several recrystallizations become fluorescent after heating to the melting-point, or owing to the influence of oxidation agents.

Provided that substances are illuminated by light of absorbable wave-lengths, the following rule will apply to solutions as well as to solid bodies: The fluorescence spectrum is independent of the *wave-length of the exciting light*. More detailed information regarding the connection between the fluorescence and the chemical constitution and the influence of the physical factors may be obtained in works by LEY, PRINGSHEIM, HAITINGER, among others.

The fluorescence of various drugs

A great deal of attention has been devoted to describing the fluorescence of various substances, including also a large number of drugs. Surveys of this kind may be found in papers by WIKANDER (1931), HAITINGER, FEIGL, SIMON (1932), RADLEY & GRANT (1935), DHERÉ (1937), HAITINGER (1938), BEQUIRISTAIN (1942), and in Handbooks of Chemistry and Physics, etc.

However, these surveys are not all in agreement. This is due to the fact that impurities may play a part with regard to the fluorescence and pure preparations have not always been used. Furthermore, it is of importance whether the substance has a crystal, powder, or liquid form. Since this has not invariably been stated in the existing surveys, the divergencies become quite explicable. Nevertheless, data conform more often with regard to some other substances, viz. generally to those with the strongest fluorescence. Still, it is hardly worth while to give a detailed account of these investigations since every scientist, wishing to make use of the fluorescence of a substance for some purpose or other, must as a rule examine the fluorescence of the compound himself. This is all the more evident in view of the particular heating technique employed,

which will be subjected to a description in Chapter III. By this means, not only the production of stronger fluorescence has been made possible, but also fluorescence in substances where it has been otherwise lacking. In Table 2, a survey will be found of some of the more common drugs which have been tested by the present author.

CHAPTER III

The detection of drugs in tissue sections by means of fluorescence microscopy

Previous investigations

In order to be able to make use of the fluorescence of the drugs for localization in tissues, it follows, as a matter of course, that the auto-fluorescence of the tissues has to be known. A large number of works exist regarding the appearance of the tissues in the fluorescence microscope.

They have shown that the tissues have an intense fluorescence of their own which is so differentiated that an examination of the sections can be performed without difficulty. Thus, staining is unnecessary for the localization of the required substances.

Thanks to this, fluorescence microscopy is suitable as a histo-pharmacological method even from a histological point of view.

With the aid of the freezing-drying method for fixation in connection with a fluorescence microscopical examination of the tissues (F. SJÖSTRAND), fluorescent substances can be carefully localized *within* the cells. This has not been possible with the earlier technique of fluorescence microscopy. The normal histological picture of a freezing-vacuum-fixed material has been described by F. SJÖSTRAND (1944) who has, in addition, summarized the most important data from earlier literature in this respect.

A great number of works also exist dealing with staining by means of fluorescent dyes (i. e. fluorochromes) and intravital microscopy with the fluorescence microscope, to which ELLIN-

GER, in particular, has devoted considerable time and attention. He has made a useful summary of these investigations (1940).

As already briefly mentioned, the fluorescence of drugs has been employed earlier for the localization of drugs in the tissues. The number of investigations on this subject is fairly restricted, as is the case with all works which may be defined as histo-pharmacological.

No studies of this kind are reported in HAITINGER'S monographs. ELLINGER'S survey of the fluorescence microscopy in biology (1940) contains only three, by v. JANCZO, FISCHL & SCHWENK and BOCK & OESTERLIN.

The first attempts to ascertain drugs in the tissues were performed by TURCHINI (1926) who contended that quinine-HBr and Na-salicylate should be recognizable owing to their fluorescence. He reported that after the injection of large doses of Na-salicylate an accumulation of this substance was detectable in the tissues of the joints. JOSEPH (1927) used the same substances with approximately identical results. He also experimented with esculin and hydrastinine. Esculin has been administered in vascular diseases and JOSEPH considered himself able to observe this substance in the intima and media of the vessels but not in the endothelium. He believed himself also to be in a position to confirm the conception that hydrastinin affects the uterus via the nervous system owing to the blue fluorescence of nerve centres, muscles and the musculature of the uterus.

CARNOT and COQUOIN (1926) also attempted to localize Na-salicylate by means of its fluorescence. They subjected photographs of the fluorescence of organs treated with Na-salicylate to comparisons with photographs of animals not treated in this way. However, they did not employ a microscopical method.

All the above-mentioned authors emphasize the difficulty of distinguishing the fluorescence of drugs (all fluorescent in blue) from that of tissues. The results obtained by them must undoubtedly be regarded as fairly uncertain.

SPITZER and Loos (1934) followed the diffusion in the central nervous system of fluorescent dyes, such as thioflavin S, coriphosphin, etc., after injections in the sciatic nerve on rabbit. They also tested novocain in this connection. This substance was evidently not ascertainable by its auto-fluorescence. Thus, the authors stated that it diminished the auto-fluorescence of the nerves, while simultaneously changing the structure of the interstitial tissue which had a swollen appearance. However, it seems open to discussion whether in this case the effect was not due rather to the water in which the novocain had been dissolved than to the novocain itself. Furthermore, also the sciatic nerve of the other side was found to be stained by the injected substance. The conclusion was drawn that the diffusion took place in the lymph tracts. Trypaflavin, etc., on the other hand, did not extend beyond the limit of the injection pressure and the diffusion. In 1941, HAAS confirmed these results by means of a similar investigation with coriphosphin, using intramuscular injection on frog.

OBERDALHOFF (1939) discovered siliciferous dust in the lungs after staining with auramin by means of fluorescence microscopy. SOFUE (1940) attempted to study the diffusion of quinine derivatives in the organs and tumours of rat. He employed ordinary frozen sections and observed them macroscopically.

MIESHER (1941) followed the penetration of fluorescent substances through the skin, using aurophosphin and eosin. Among real drugs, only trypaflavin and salicylic acid was applied, particular attention being paid to the difficulty of following this substance owing to the fact that the tissues fluoresce in the same colour.

GRAFFI (1939—41), GÜNTHER (1941—42), DONIACH, MOTTRAM and WEIGERT (1943), among others, have studied the penetration of benzpyrene and other cancerogenic substances through the skin and their localization.

The attempts to localize vitamins are numerous and, particularly concerning the A and B vitamins. VON QUERNER (1932, 1935) and VON QUERNER and STURM (1934) observed

a fluorescent substance in liver cells, retina, fresh liver oils and butter which they considered to be an A vitamin. These investigations were later repeated by HIRT (1939) who obtained on the whole similar results. Also PATZELT and SHAIRER (1940—41) and POPPER (1940) have performed examinations with A vitamins.

ELLINGER and KOSHARA (1933, 1934) identified a yellow-coloured fluorescent substance in the liver and in the kidneys as riboflavin. VON EULER, HELLSTRÖM and ADLER (1935) studied the occurrence of riboflavin in the eye of the cod and some mammals. ELLINGER and HIRT (1938 and 1939) followed riboflavin through the kidneys and the liver. Apart from the work by v. EULER and col., no definite reasons have been offered proving the examined substances to be actually vitamin A or B.

Also the relation of chemo-therapeutics to bacteria and trypanosomes has been studied by means of fluorescence microscopy. Thus, v. JANCZO' (1932) examined the effect of acriflavin, trypaflavin, and other chemo-therapeutics on trypanosomes. The drugs were injected into infected mice and rats. In the blood of treated animals the trypanosomes disclosed marked fluorescence. However, resistant strains remained unstained and the conclusion was drawn that in order to cause an effect these drugs must penetrate into the parasites.

Similar experiments were described by FISCHL and SCHWENK (1932). FISCHL and SINGER (1935) were able to prove that atebrin penetrated into the malarian plasmodia on birds, while BOCK and OESTERLIN (1939) ascertained atebrin and quinine in the malarian plasmodia of monkey.

FR. FALKENSTEIN (1932) subjected a rabbit uterus to treatment with flavadin (which was used earlier in cervix gonorrhoea). He found that both the mucous membrane and glands fluoresce in a greenish-yellow colour owing to flavadin. These greenish-yellow streaks extended right down into the muscularis mucosæ. HOFSTÄTTER (1938) repeated these experiments.

Finally, HELANDER, RICHTNÉR, F. SJÖSTRAND and T. SJÖSTRAND have examined the resorption of sulfathiazole through the mucous membrane of the nose on rabbit (1945).

Own investigations

The fluorescence of heated drugs

Drugs with a fluorescence in colours other than blue, e. g., red, green, yellow, brown, etc., are, as a rule easily detectable in the tissues, as mentioned earlier.

As has already been pointed out, the fact that the blue fluorescence of drugs is surpassed by the auto-fluorescence of tissues represent, on the other hand, one of the most difficult problems in histo-pharmacological studies by means of fluorescence. Therefore, an attempt to change the conditions of fluorescence so as to bring forth a contrast was looked upon as urgent. Various methods of elucidating this question were tested. Attempts were made to eliminate or reduce the fluorescence of the tissues by illumination with ultra-violet light. The effect of prolonged illumination is extremely strong, causing marked reduction in the fluorescence of the tissues. Unfortunately, however, the same reduction occurs in the fluorescence of the drugs. Attempts were performed to treat the tissues with the above-mentioned substances (Chapter 2) for reducing fluorescence but without avail. Since every method involving a possibility of diffusion in the tissues should a priori be avoided, the question whether or nor heating of the slices causes any change in the fluorescence colour was tested in the hope that drugs would be more resistant than tissues towards heating. In the following, an apparatus will be described which has been constructed with a view to obtain an even temperature of the whole slide.

Apparatus. A small electrical furnace (Fig. 2) was used for heating the specimens, the construction of which is shown in Fig. 3. The joints of the furnace chamber are welded. The door can be made perfectly airtight when so required. At the rear, a tube is inserted into the wall for

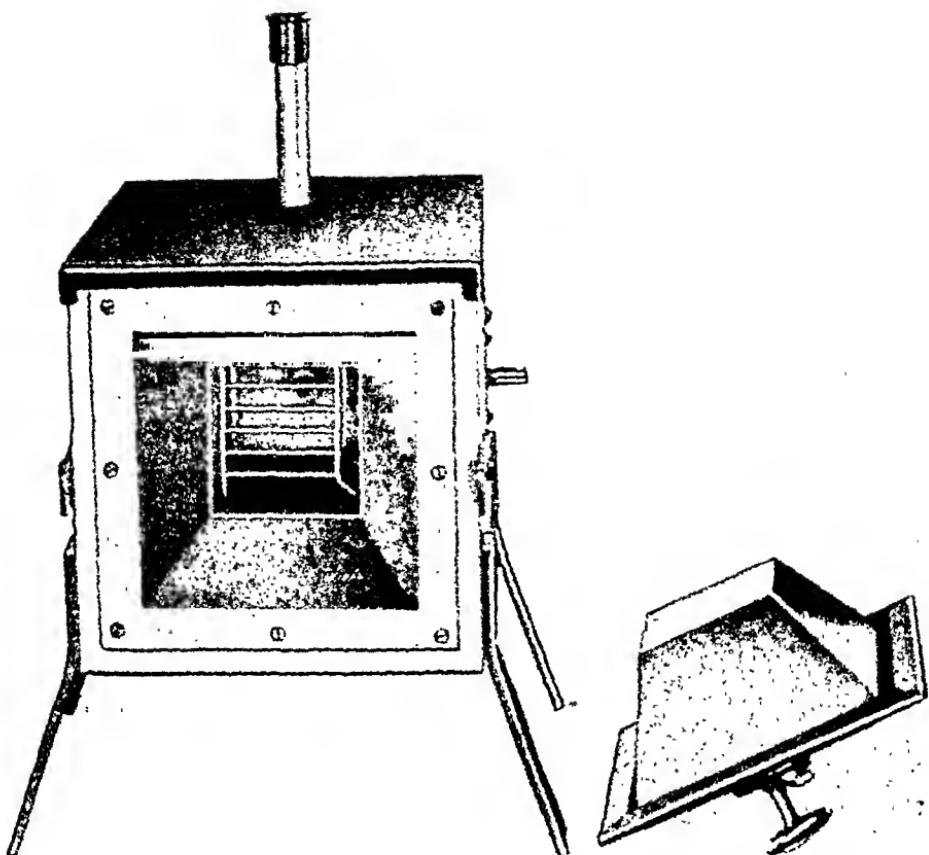


Fig. 2. *The electrical furnace for heating sections at a constant temperature.*

connection with the outer air, this to facilitate heating when necessary without the afflux of air, in the presence of other gases, etc. The temperature can be modified by means of a mercury thermometer-thermostat which connects or disconnects the current according to the particular temperature at which it has been set. As a rule, a thermometer adjustable at a temperature of up to + 300° C is adequate for this purpose. The temperature may be maintained at a sufficiently constant degree by means of this regulation contrivance. At +200° C the variations in temperature amount to a maximum of $\pm 5^{\circ}$ C.

Na-salicylate was used, to begin with, in these investigations, this substance having long been well known for its pronounced fluorescence. The very first experiments with musculature, injected with Na-salicylate, indicated the probable usefulness

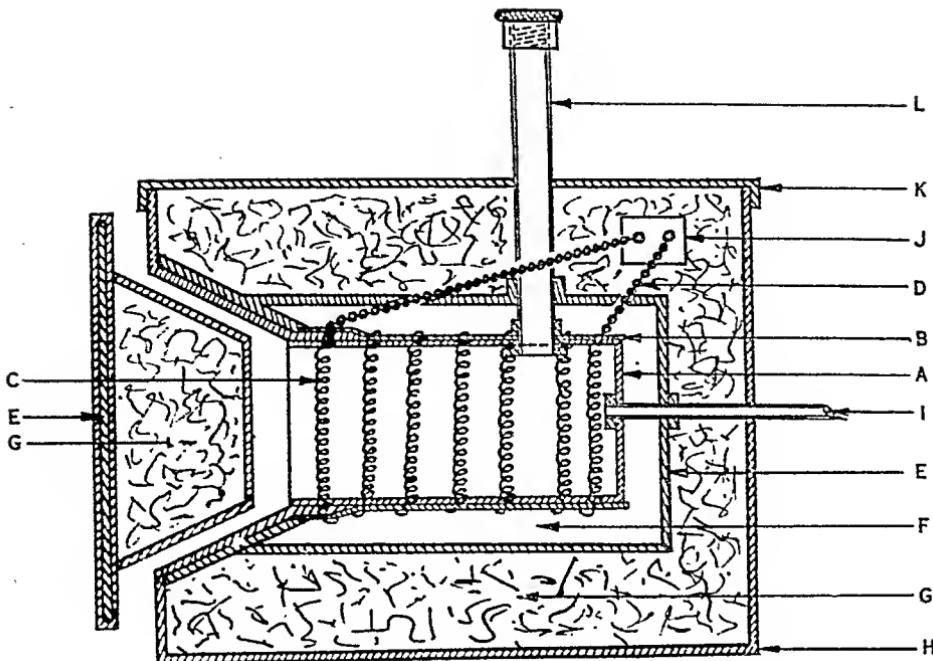


Fig. 3. A diagram of the furnace.

A = Copper wall of furnace chamber.
 B = Mica insulation.
 C = Chromium-nickel thread.
 D = Insulated wire leading to plug connecting with 220 volt a. c.
 E = Asbestos sheet.
 F = Air space.

G = Asbestos powder.
 H = Iron plate.
 I = Pipe for evacuation of furnace when required.
 J = Eternite slab.
 K = Removable lid.
 L = Tube for thermo-stat.

of this method. Although the fluorescence of the tissues had not admittedly disappeared after heating up to $+200^{\circ}\text{C}$ for 5 minutes, as had been expected, a change had, nevertheless, occurred to a yellow colour, while the Na-salicylate in the tissues remained a blue-violet. Later experiments with sulfathiazole-Na disclosed a change in the colour of this substance too, after heating for the purpose of obtaining a contrast with the fluorescence of the tissues. At 225° for 3 minutes, the tissues became yellow and the sulfathiazole brown. Gradually, a still more distinct contrast appeared at 170° 5 min., the tissue still being a blue-grey and the sulfathiazole a clear yellow. These colours remain even after the cooling of the slices.

Another fact of great significance was the considerably more intense fluorescence of the tissues after heating to a temperature of between 150° and 225° , than before the heating process. Accordingly, the examination of the sections was facilitated (HELANDER 1943).

The fluorescence of tissues after heating

As a result of this observation, the condition of the tissues and some drugs, at heating to different temperatures, was subjected to more systematic analysis.

Incineration of histological sections has long been performed in histo-chemical examinations, particularly as far as the studies of the inorganic constituents in the cells are concerned. On the other hand, no observations seems to have been made regarding the reaction of the cells and the tissues at heating to different temperatures for varying periods of time. And yet, these conditions should be of a certain interest even *per se*, apart from their significance with regard to the usefulness of fluorescence microscopy in histo-pharmacology. For the tissues change their fluorescence colour in an exceedingly regular fashion at increasing temperature and when exposed to a prolonged period of heating, different elements acting differently in this respect.

Generally speaking, it may be said that the usual change in colour at an increase of temperature is as follows (the data below concern a heating time of 5 minutes): The general, rather dark blue auto-fluorescence of the tissues is transformed to a lighter blue at $+150^{\circ}$ C, becoming blue-grey at 175° , grey-yellow at 200° , grey-yellow-brown at 225° , yellow-brown at 250° and dark brown at 275° . At 300° they lose their fluorescence and appear quite dark. However, there are quite a few exceptions from this general rule, only a small number of which will be mentioned here. (The changes in colour of various tissues and cells will be found in Table I.)

Elastin is distinguishable from other tissue constituents owing to its intense blue colour. This colour is, in addition, very thermo-resistant. It remains even after heating at 225°

Table 1. *The fluorescence colour of various tissues after heating to different temperatures.*

Structures and Tissues	Before heating	150°		175°		200°		225°		250°		300°	
		5 min.	3 min.	5 min.	3 min.	5 min.	3 min.						
Elastin	bl.	bl.	p. bl.	p. bl.	p. bl.	p. bl.	p. bl.	p. bl.	p. bl.	p. bl.	bl.-w.	g.-w.	y.-w.
Collagen	d. bl.	d. bl.	d. bl.	d. bl.	d. bl.	d. bl.	d. bl.	d. bl.	d. bl.	d. bl.	p.-g.	p.-g.	0
Fat tissue	bl.	g.-w.	y.-g.-w.	g.-y.	g.-y.	0							
Muscles, skeletal	bl.-w.	bl.-g.; w.-g.	g.-br.	g.-br.	0								
" , in skin	y.-g.	y.	d. y.-g.	d. y.-g.	0								
" , cardiac	bl.-g.	bl.-g.	bl.-g.	bl.-g.	bl.-g.	bl.-g.	bl.-g.	bl.-g.	bl.-g.	bl.-g.	y.-br.	y.-br.	0
" , smooth	bl.-g.	y.-g.	g.-y.	g.-br.	d. g.-br.	0							
Nerves		Intense bl.	bl.-w.	g.-y.	g.-y.	0							
Ganglion-cells	cytopl.	p. bl.-g.	p. g.	w.-y.	w.-y.	0							
" , nuclei	nuclei	d. bl.	g.-w.	g.-w.	0								
Resp. epithel. cells	cytopl.	g.-w.	d. g.	p. g.	y.-w.	y.-w.	0						
Kidneys tubular cells	cytopl.	bl. and g.	d. y.-g.	d. y.-g.	0								
Spleen cells	cytopl.	nuclei	d. bl.	g.-y.	g.-y.	0							
Liver cells	cytopl.	granula	y.	y.-br.	g.-y.	g.-y.	0						
Intestine cells	cytopl.	nuclei	d. bl.	p. bl.	g.-w.	g.-w.	0						
Red corpuscles		0	0	d. g.-br.	br.-y.	br.-y.	0						

List of abbreviations:

bl. = blue

g. = grey

y. = yellow

d. = dark

br. = brown

w. = white

0 = no fluorescence.

p. = pale

for 5 minutes. Consequently, elastin is always easily distinguishable from other elements in the tissues. The same applies to collagen which has, nevertheless, a considerably darker colour.

The cellular nuclei vary somewhat in the different organs. While the nuclei in renal tubules, for instance, are grey blue at 175°, the cellular nuclei of the liver have a pale blue colour. This must, in all likelihood, be ascribed to a difference in the cellular constitution. However, both become grey-brown at a high temperature. The yellow granules in the reticulo-endothelial system lose their fluorescence at approximately 175°.

The ganglion cells of the brain also disclose specific conditions, being almost white, while other cellular nuclei are grey-yellow or yellow at 200—225°. Nevertheless, since an analysis of the reasons for these changes in colour would not be of any particular interest in this connection, there is hardly any reason to enter more closely upon these problems.

Within certain limits the same effect can be achieved either by an increase in the temperature or by a prolongation of the heating. Therefore, in the following it has generally not been considered necessary to vary the heating periods but merely the temperature. See further Table 1.

The detection of drugs in tissues after heating the sections

The possibility of detecting drugs in the tissues by means of this method has been investigated as follows: Drugs have been injected in 1 or 0.1 per cent solutions into the femoral musculature of mice at doses of 0.2—0.5 ml. Large parts of the musculature have been soaked with the fluid. After 15—30 minutes, the animal has been killed and about ten muscular pieces (from different parts of the musculature) have been excised and prepared by means of the usual freezing-drying method.

Then, the sections have been heated to different temperatures, the standard series of 150°, 175°, 200°, 225° and 250° C having been used for periods of 5 minutes (one slice for each

temperature) and subjected to examination in the fluorescence microscope. This has been done in order to establish whether sufficient contrast was obtainable in the tissue. As may be derived from Table 2, it was found that several drugs were ascertainable in this way, owing either to a change in their colour or to the fact that they maintained their colour while the tissues changed theirs. It is noteworthy that in some cases, e. g., acetylcholine, substances without fluorescence have obtained this property after heating. As may be seen from the table, the most common transformations in colour are those from yellow to brown.

The reason for this change in colour has not been closely inquired into. In some cases, an oxidation of the cellular structures has probably taken place, in others a re-arrangement of the molecular structure of the substances may be conceivable. Nor may the fact be overlooked, that the adsorption or proximity to the tissue structure may, sometimes be of significance with regard to the change.

Accordingly, as regards sulfathiazole, the change in colour will, at least in part, be due to the oxidation of the drug. Thus, it has been demonstrated that a similar change in the fluorescence was obtainable by boiling sulfathiazole together with hydrogen peroxide.

In the fore-mentioned work by S. DUTT (1930), a similar result was arrived at, viz. that substances (quinine, eosin, fluorescein), which had lost or decreased their fluorescence during repeated recrystallizations (18—58 times during the course of seven years), regained their fluorescence after treatment with oxidation agents or by heating to the melting-point for a short space of time (i. e. approximately 10 minutes).

However, in this connection the principal matter is that changes in colour actually take place. Accordingly, the cause of the changes has not been studied in other instances.

The concentration limit obtained after heating the substances may differ to some extent. In general, a concentration of at least 1: 1 000 of the various drugs can be detected. The concentration limit of sulfathiazole has been determined in

Table 2. *The fluorescence colour of various drugs and*

Drugs	Melting points	Fluorescence in pure, pulverized form		Fluore-	
		Colour	Intensity	Before heating	Contr.
Ascorbic acid	190—2°	bl.	+	—	—
Acetylcholine Roche	172—3°	p. bl.	++	—	—
Atropine sulf.	181—3°	bl.	++	—	—
Dicodide bitartrate	146—8°	gr.	+++	—	—
Dionin	125°	y.	+	—	—
Eucodal	270°	—	—	—	—
Eupaverine	158°	gr.	++++	gr.	++
Homatropine hydrobromide	212°	—	—	—	—
Pantocain	149—50°	bl.	+++	—	—
Papaverine hydrochloride	231°	v.	+	p.-gr.	+++
Penicillin	—	gr.-y.	+++	—	—
Percain Ciba	97°	bl.	++	—	—
Priscol Ciba	171°	—	—	—	—
Prontosil sol. Bayer	—	r.	+++	r.	++++
Riboflavin	275—80°	y.	+++	gr.	+++
Salazopyrine Pharmacia	—	r.-br.	+	g.-br.	+++
Salazothiazole Pharmacia	—	r.-br.	+	d. br.	+++
Sodium Salicylate	—	bl.	+++	bl.	+
Sulfanilamide Astra	163°	bl.-g.	+	—	—
Sulfapyridine Pharmacia	190—3°	bl.	+++	—	—
Sulfathiazole Astra	202°	bl.	+	—	—

The distinctness of the contrast is denoted by +. The greater the number of + signs,

List of abbreviations: bl. = blue br. = brown g. = grey gr. = green

the change in colour of tissue sections after heating.

scence in tissues								Concen- tration of injected solutions	
After 150° 5 min.		175° 5 min.		200° 5 min.		225° 5 min.			
Colour	Contr.	Colour	Contr.	Colour	Contr.	Colour	Contr.		
br.	+++	br.	+++	y.-br.	+++	y.-br.	++	1:100	
y.	+	y.	+++	y.	++	y.-br.	++	1:4000	
br.-y.	+++	br.-y.	+++	r.-v.	++++	p. r.	+	1:100	
—	—	g.-br.	++	y.-br.	+++	br.-y.	++	1:100	
br.-g.	+	br.-g.	+++	br.-g.	++	y.-br.	+	1:100	
f. y. fl.	+	y.	+++	y.	+++	y.	++	1:100	
gr.-y.	+++	g.-y.	++	g.-y.	+	p. br.	+	1:100	
f. br. fl.	+	br.-y.	++	y.	+++	y.	+++	1:100	
y.	++	y.	+++	y.	+++	y.-br.	++	1:100	
p. gr.	+++	br.-y.	+++	br.-y.	+++	br.-y.	++	1:100	
y.	+	y.-br.	+++	y.-br.	+	br.	+	1:1000	
y.-g.	+	y.-g.	+++	y.-g.	++	y.	+	1:100	
—	—	y.	+++	y.	++	y.	+	1:1000	
r.	+++	r.	++	r.	++	r.	++	1:1000	
gr.-br.	++	br.	+++	br.	+++	br.	+	1:1000	
g.-br.	+++	br.-g.	+++	br.-g.	++	g.-y.	+	1:1000	
d. br.	+++	p. br.	+++	p. br.	+++	r. br.	+++	1:1000	
bl.	+++	bl.	+++	bl.	+++	br.-g.	+	1:100	
y.	+++	y.	+++	y.-br.	++	br.	++	1:1000	
y.	+++	y.	+++	y.-br.	++	br.	++	1:1000	
—	—	y.	+++	p. br.	+++	d. br.	+++	1:1000	

the more distinct the contrast colour. See further page.

r. = red

v. = violet

y. = yellow

p. = pale

contr. = contrast.

accordance with previous methods adopted with regard to prontosil, and has been found to lie at 1:1 000—1:10 000. This concentration limit is somewhat higher than that of prontosil. Therefore, it is insufficient for the ascertainment of sulfathiazole given in therapeutical doses, provided that the distribution in the tissues is diffuse and approximately equal to the blood concentration. However, this method is adequate in the case of sulfanilamide compounds when occurring in somewhat higher concentration than that of the blood, e. g., in the kidneys, and at local application. *Thus, this method of ascertaining sulfathiazole and other sulfanilamide derivatives after heating must be regarded as quite useful for the above-mentioned purposes.*

Apparatus and photography: REICHERT'S fluorescence lamp Lux UV, with the mercury high-pressure burner Hanau S 100, has been used as a source of light for the fluorescence microscope. The optic is made of UV glass. A liquid filter, 30 mm thick, with a copper-sulphate solution (2.5 %) has been employed, as well as two filters of black glass, 2.5 mm thick.

The microscope consists of an ordinary Zeiss microscope stage with an UV glass condenser and Leitz' non-fluorescent objectives 6 Fl and 1/12 Fl. These objectives are supplied with an objective iris diaphragm which increases the distinctness of the pictures to a great extent. Ordinary objectives are used in the case of lower magnification than 20 \times . Zeiss' non-fluorescent immersion oil is applied. Lastly, an ocular filter absorbing the UV is placed on the ocular. A micro outfit (Miflex) with a Leica camera is used for photography. The Agfa-color daylight film has been chosen for the purpose. At low magnification, i. e. to 20 \times and lower, the ocular filter absorbing UV has been applied, while no filters occur when the objectives 6 Fl and 1/12 Fl have been made use of (these objectives having in themselves an euphos glass filter). The times for exposure have varied from 5 to 45 minutes. Owing to the fact that different film rolls may have somewhat varying degrees of sensitivity, that varying lengths of exposure are required in order to do justice to the different colours, and, further, since an estimation of the strength of light of the slice concerned is extremely difficult, as a rule, 3 to 4 pictures have been taken of each slice with varying times of exposure. It should be noted in this connection that new sections or new parts of the sections have to be taken at each exposure, on account of the intense reduction of the fluorescence of the slice when exposed for longer periods of time.

PART II

PROBLEMS AND RESULTS

CHAPTER IV

Aspects of the problems and earlier investigations regarding the relationship between the effect and localization in the tissues of sulfanilamide derivatives

The therapeutical effect of sulfanilamide as compared to prontosil rubrum and soluble

Animal experiments

The first sulfanilamide derivatives produced by KLARER and MIETSCH, the effect of which was demonstrated by DOMAGK on experimentally infected animals, were prontosil rubrum and prontosil soluble. The former substance is a basic azo-dye¹ and the latter one an acid azo-dye. DOMAGK proved prontosil to be inactive in vitro, its chemo-therapeutical effect being manifested only in vivo. His finding was confirmed by LEVADITI and VAISMAN (1935) and, a little later, by NITTI and BOVET (1935). TRÉFOUËL, TRÉFOUËL, NITTI and BOVET (1935) found that the sulfanilamide contained in these two compounds had a chemo-therapeutical effect in vivo, as well as in vitro. They concluded that, in all likelihood, the prontosil was converted to sulfanilamide in the organism. These results were soon confirmed by several authors, viz. COLEBROOK, BUTTLE and O'MEARA (1936), COLEBROOK, KENNY (1936), BUTTLE, GRAY, STEPHENSON (1936), LONG, BLISS (1937). In 1938, ENGEL demonstrated the reduction in vitro of prontosil to sulfanilamide caused by liver, kidney and blood. Later, many at-

¹ As regards the formulae, see the folded page at back of cover.

tempts have been made to prove that prontosil only produces an action after conversion to sulfanilamide.

In 1937, FULLER found that approximately half of the administered dose of prontosil was converted. However, he was unable to prove that this quantity would suffice to explain the effect on infected animals. In 3 groups comprising 10 infected mice who had been administered, on an average, 25 mg of prontosil soluble parenterally, 7, 7 and 5 mice, respectively, had died by the 5th day. 6 out of 10 mice, which had received 22.5 mg of sulfanilamide, had died on the same day. In this case, the preparations had produced about the same effect, notwithstanding the fact that, according to FULLER'S investigations in this particular instance, only approximately $\frac{1}{6}$ of the injected prontosil dose can have been in the form of sulfanilamide. He states further that the infected groups receiving prontosil excreted more sulfanilamide than did the normal groups.

FEINSTONE, BLISS, OTT and LONG (1938) compared the effect of prontosil soluble and sulfanilamide after administration in one single peroral dose. They employed equimolecular doses and achieved almost the same effect with both preparations. However, in order to render these experiments comparable, it is essential that regard be paid not only to the necessity of equimolecular doses, but also to the precise quantity which has been reduced. Therefore, the blood concentration of sulfanilamide has been determined after the administration of prontosil soluble, as well as pure sulfanilamide, in doses of 20.5 mg and 6 mg, respectively.

The result then obtained was somewhat surprising, i. e. *all* the prontosil had been converted to sulfanilamide, and the same blood concentration occurred (in mg per cent), whether 1, 2, 3 or 4 g were injected of prontosil soluble per kg of body weight. Since a large quantity of the injected prontosil dose is observable as excreted into the urine in an unchanged condition, the possibility of some source of error inherent in the method of determining the sulfanilamide may be considered. The above-mentioned authors have, for instance, not offered any explanation of the manner in which the sulfanilamide has

been determinable in the presence of prontosil, which is strongly coloured. In another work by LONG and BLISS (1938), they state that from 65 to 75 per cent of ingested prontosil is reduced in the body.

Also LITCHFIELD, WHITE and MARSHALL (1941) establish the fact that the chemo-therapeutical effect of prontosil is altogether dependent on the sulfanilamide formed. They draw attention to the inadequacy of earlier investigations, with a view to demonstrate the effect of prontosil as due to the conversion to sulfanilamide performed by GLEY, GIRARD (1936), BUTTLE, GRAY, STEPHENSON (1936) and FEINSTONE, BLISS, OTT, LONG (1938). These authors based their conclusions merely on the effect of a single daily dose. This would not suffice considering the difference between the blood concentration-time curves of the two drugs. LITCHFIELD, WHITE and MARSHALL instead adopted the principle of mixing the compounds with the food. The drugs were administered in equimolecular quantities, 0.1 per cent for sulfanilamide and 0.4 per cent for prontosil. In this way, they obtained comparable blood concentration-time curves during a series of twenty-four hours. They also discovered that all prontosil was converted to sulfanilamide. Nevertheless, the fore-mentioned criticism applies also in this case. For quite a large amount is, all the same, excreted in the form of prontosil.

However, since the effect of equimolecular doses of prontosil soluble and sulfanilamide on infected animals in their investigations was approximately similar (in both cases), they inferred that the chemo-therapeutical activity of prontosil is dependent on the sulfanilamide formed by it.

Still, whether or not the effect of prontosil and sulfanilamide is equal in all kinds of infections is not deducible from these experiments. They merely serve to show that the blood concentration is a determining factor with regard to the result of the treatment of sepsis, animals without a positive blood culture at the heart puncture having been excluded. In addition, it has not been definitely proved that all the prontosil has been converted into sulfanilamide. In fact, the results arrived at by

several other investigators appear far more probable, viz. that about half of the administered dose of prontosil soluble is reduced. Nor have the drugs been shown to have an equal effect on infections of a more local nature.

The coupling to an azo-dye has, on the basis of these results, rather generally been concluded to be unnecessary and insignificant with regard to the chemo-therapeutical effect. However, DOMAGK, among others, calls in question the accuracy of these statements, declaring that in several instances the effect of coloured prontosil compounds is better than that of sulfanilamide.

This belief in the superiority of prontosil preparations to sulfanilamide in some infections finds support in animal experiments, as well as in clinical experience. In a typical test series, published by DOMAGK in 1944, mice in groups of 20 were subjected to intramuscular infection with streptococci and treated after periods of 1, 8 and 24 hours, respectively. None of the 18 controls survived, and only 4 of the ones treated with sulfanilamide and 8 of the ones given prontosil soluble. It should also be noted that the same doses in grammes have been used in the case of both drugs. Nor has any regard been paid to the fact that equimolecular doses would have required about 3 times as large a dose of prontosil. Further, probably only half the prontosil dose is converted to sulfanilamide. As already mentioned, FULLER arrived at a similar result. In 1937, WHITBY produced approximately the same effect by the subcutaneous injection of 7.5 mg of prontosil soluble and an oral administration of 25 mg of sulfanilamide on mice infected with streptococci.

Clinical examinations

A disease which has been particularly made use of clinically for comparisons between prontosil soluble and prontosil rubrum, on the one hand, and sulfanilamide, on the other, is erysipelas. Still, data regarding the clinical experiences are very hard to judge, owing to the extremely incalculable

course of this particular disease and its marked tendency towards self-healing. Moreover, published investigations are, especially with regard to the description of the primary material, frequently too restricted to afford any facilities for determining with certainty, in the first place, whether chemo-therapeutics have any better effect at all than earlier methods of treatment and, in the second place, whether any of the chemo-therapeutics employed have been superior to others. In addition, owing to the fact that at first exceedingly small doses have often been given and data regarding the dosage are lacking in some instances, and by reason of the confusion caused by using the term prontosil album for Bayer's sulfanilamide preparation, and, finally, because of the frequent neglect to specify the particular prontosil preparation employed (simply prontosil being referred to), it is very difficult to find any comprehensive material in the literature containing direct comparisons between the coloured prontosil compounds and sulfanilamide.

As early as in the year 1935, a number of works existed dealing with the good effect of the coloured prontosil derivatives on erysipelas (KLEE and RÖMER, GMELIN, SCHREUS). SCHREUS even goes so far as to consider these drugs as a specific means to counteract this disease. He is of the opinion that this can, to a large extent, be explained by the staining of the skin caused by these drugs. Favourable results with coloured prontosil preparations have also been obtained by TONNDORFF (1936), BECKER (1937), MEYER ZU HÖRSTE (1936), UNSHELM (1937), SNODGRASS and ANDERSSON (1937), BUCHHEIM and DRECHSLER (1938), STRÖBEL (1939), ANDERSON (1939), VOLAVSEK (1940), LÖHE (1940), HOFFHEINTZ (1940) and HEGLER (1944).

In a large statistical survey of 998 cases, 162 of which were treated with sulfanilamide, HOYNE, WOLF and PRIM (1939) found this method of treatment superior to those used earlier. This result was obtained also by SNODGRASS and ANDERSSON (1938), TOOMEY (1938), CHRISTENSEN (1939), NELSON, RINZLER and KELSAY (1939), FOLEY and YASUNA (1940),

and others. Thus, it must be looked upon as fully established that chemo-therapeutics are superior to the methods of treatment used earlier for erysipelas. The small number of authors who have not found any effect with chemo-therapeutics have based their investigations on very restricted materials, containing as a rule only about thirty cases. In addition, they have generally applied this form of treatment at a fairly late phase in the course of the disease. [HARTL (1936), KÖNIG (1939), ISHIKAWA (1939) and SITTENAUER (1940).]

The investigations performed with the direct purpose of comparing the effect of coloured prontosil preparations and sulfanilamide are rather few. POLONY (1939) concluded, after having treated approximately 150 cases of erysipelas, that the coloured chemo-therapeutics were more favourable than sulfanilamide. SNODGRASS, ANDERSSON and RENNIE (1938) compared the effect of prontosil rubrum, sulfanilamide and benzyl sulfanilamide on a series of 242 cases in all. In an earlier examination of 312 cases, treatment with prontosil rubrum was found to be more effective than with ultra-violet light. The same applies to sulfanilamide in relation to ultra-violet light in another series of 270 cases. In these examinations, the average total dose of prontosil rubrum was 5 g, the maximum dose being 15 g. The corresponding figures as regards sulfanilamide are 14.64 g and 41.6 g.

In the comparative examination between prontosil rubrum and sulfanilamide, the doses were approximately equimolecular, 1—2 g of prontosil rubrum being administered every 4th hour and 0.5—0.75 g of sulfanilamide every 4th hour. Prontosil rubrum was but slightly superior to sulfanilamide. Nevertheless, it should be emphasized that the results are still not quite comparable. A proper comparison would require a conversion of all the prontosil to sulfanilamide. However, such a complete conversion does not occur. The fact that the toxic effects on a patient treated with sulfanilamide are about twice as frequent as those on patients given prontosil may also serve as further indication of the but partial reduction of the prontosil. Thus, taking for granted that prontosil exerts its effect by

reduction to sulfanilamide, the same result will be obtained with prontosil as with sulfanilamide, though without the toxic effects in the former case. In my opinion, this must be interpreted as a concentration of sulfanilamide, produced by reduction of prontosil, at the site of the infection at least equal to the one obtained with sulfanilamide, though with a lower blood concentration in the former case.

It is, in fact, a usual mistake to suggest that sulfanilamide and other simple sulfanilamide derivatives produce effects identical with those of prontosil, the same dosage or even a lower dose of the coloured prontosil preparations having been administered, without any regard to the fact that the doses are not equimolecular and that not all the prontosil has been reduced. Since the same effect has been ascertained with sulfanilamide as with prontosil soluble, with a similar dosage in grammes, and the blood concentration of sulfanilamide after prontosil treatment may, in all likelihood, be assumed to amount, at most, to $\frac{1}{6}$ of the one obtained after administration of pure sulfanilamide, prontosil must in these cases be said to be superior to pure sulfanilamide. These observations may also serve to throw light upon the insignificant toxic effects produced by coloured prontosil preparations.

The reason why coloured prontosil preparations are, in some cases, superior to pure sulfanilamide is unknown. LEVADITI (1938) has tried to account for this discrepancy between dose and effect by assuming that the prontosil preparations, as well as so many other dyes, are absorbed by the R. E. S., where they attain high concentrations. This explanation would be all the more appropriate in view of the fact that even bacteria are usually phagocytized in these cells. Accordingly, the prerequisites for a chemo-therapeutical effect (i. e. a contact between sufficiently high concentrations of chemo-therapeutics and bacteria) would increase by the azo linkage.

Still, this is merely a conjecture, no relationship having been demonstrable as between the R. E. S. and prontosil compounds. BORELL and TROELL (1943) showed that sulfathiazole did not hinder the capacity of the R. E. S. to absorb trypan blue.

The investigations of LEVADITI, BEQUIGNON & REINIÉ (1938) have been carried out as follows: They subjected mice to intra-peritoneal injection of carbon, tubercle bacilli, etc. After a few days, when inflammatory granuloma had developed in the abdominal cavity, prontosil rubrum was administered per os. The granuloma were seen to have a red colour. The microscopical examination revealed granules of a red substance (since the fixation method has not been specified, the data regarding the localization are difficult to judge). Thus all that has been proved is that prontosil may be observed in the inflammatory connective tissue. However, DOMAGK regards this hypothesis as a possible explanation, but is not convinced that the prontosil exerts its effect only by the reduction of sulfanilamide.

He writes as follows: »The fact that only part of the azo compounds are converted reductively does not explain why the effect of the azo compounds on infected animals, as well as on streptococcal infections in human beings, is often considerably better than that corresponding to the amount of reductively converted sulfanilamide».

Thus, since a difference obviously exists between the effect of sulfanilamide in a pure form and that of sulfanilamide coupled to an azo-dye, though as yet not elucidated, an examination seems justified regarding the difference, if any, in the localization between the two types of drugs, considering that the concentration in the infected tissues, and not the blood concentrations, determines the therapeutical effect.

Earlier investigations regarding the distribution of sulfanilamide derivatives in the tissues

Many authors have examined the distribution of unstained sulfanilamide derivatives in the tissues and in the fluids of the organism by means of chemical methods. As a rule, the same methods for the ascertainment of these compounds which are used on fluids, have, in principle, been employed on tissue extracts, i. e. the diazotized sulfanilamide derivatives are

coupled to aromatic amino groups. Such methods have been described by several authors. The method most often resorted to is, no doubt, the one propounded by MARSHALL, EMERSON and CUTTING (1937) which has later been modified and improved by, among others, BRATTON and MARSHALL (1939), MAHER and CAMP (1938), SIMESEN (1939) and RICHTNÉR (1943).

Some of the results obtained have been summarized in Table 3. In this way, of course, only a survey will be obtained of the manner of distribution of the sulfonamides, owing to the variation in the methods adopted and the heterogeneous character of the materials, which have often also been too restricted for statistical analysis. The animal species seems to be of particular significance for the concentration in the cerebrospinal fluid and in the red blood corpuscles. Above all, the fact that the tests have been performed at different times after the administration of the substances has, undoubtedly, frequently involved a divergency in the results.

However, some of the conclusions which have been drawn appear to be well founded. Thus, the sulfanilamide has a very even distribution in the whole organism, with the exception of the adipose tissue and the skeletal system, where the concentration is stated to be somewhat lower, and in the kidneys and the liver where they exceed the blood concentration. Sulfa-pyridine in the tissues, as a rule, does not amount to more than approximately 65—90 per cent of the blood concentration, being still somewhat lower in the brain and the cerebro-spinal fluid where the concentration equals 50—80 per cent of the blood concentration in man and 30—40 per cent in rabbit. Sulfathiazole is found to have the lowest concentrations in the tissues, being 20—50 per cent of the blood concentration, with the very lowest concentration in the brain.

As far as I have been in a position to establish with regard to prontosil, only examinations of the conditions in the cerebro-spinal fluid exist. This fluid does not under normal circumstances, contain any of these substances (VONKENNEL and SCHMIDT, 1939). Moreover, the greater skin affinity of pronto-

Table 3. *A survey of earlier chemical investigations in tissues and*

Year of publication	Authors	Species	Sulfanilamide								
			Cerebrospinal fluid. Brain	Red corpuscles	Kidney	Adenoid	Lung	Liver	Muscle	Skin	Bone, fat.
1943	Alexander	Rabbit	6	—	23.3	—	—	11.8	8.7	—	—
1944	Domagk	Mouse	6.5	— ¹	9	—	8.4	8.4	11.5	—	—
1943	Frisk	Man	—	>10	—	—	—	—	—	—	—
1944	Herberts	Rabbit	—	—	—	—	—	—	—	—	—
1937	Marshall & al.	Dog	7.5	—	—	—	10	10	10	8	2-3
1941	Simesen & al.	Man	—	>10	—	8	—	—	—	—	—
1942											
1941	Strauss & al.	Man ²	7-8	—	7.5-9	—	6-10	7-10	—	—	—

The figures (6) denote the ratio

$\frac{\text{concentration of free drug in the tissue}}{\text{concentration of free drug in the blood}}$.

The figures (6) denote the ratio

$\frac{\text{concentration of total drug in the tissue}}{\text{concentration of total drug in the blood}}$.

¹ Man.

² Post mortem.

*regarding the concentration of various chemo-therapeutics
body fluids.*

S u l f a p y r i d i n e							S u l f a t h i a z o l e										
Cerebrospinal fluid.	Brain	Red corpuscles	Kidney	Adenoid	Lung	Liver	Muscle	Pleural effusions	Cerebrospinal fluid.	Brain	Red corpuscles	Kidney	Adenoid	Lung	Liver	Pleural effusions	Hours after i. v. injection
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3
6.6-8.6	8-9	—	—	—	—	—	—	—	10	1.3-3.2	5	—	—	—	—	—	10
2.2-5.5	4	—	—	—	8.9	8.7	6.3	—	—	—	2.7 ¹	—	—	—	—	—	1/2
5.5-8.3	>10	—	8	—	—	—	—	—	1.2-2.2	3	—	—	5	—	—	—	—
6-9	—	10-30	—	5-10	5-14	—	—	3-4	—	20-30	—	—	—	8-11	6-19	—	—

Blood-concentration = 10.

sil rubrum than of prontosil soluble has been pointed out. The latter assumption has been based on the different degree of staining of the skin which has been observable with the various drugs (BOSSE, 1938).

The unstained sulfa-preparations occur in the same concentration as that of the blood in pleural effusions. The longer the time elapsing between the administration of the dose and the examination, the more even is, as a rule, the distribution of these unstained sulfanilamide derivatives. The fact that the kidneys and, in some instances, the liver have a higher concentration than that of the blood (TAYLOR, ÅGREN, 1940, and others) is due to the excretion of these substances in the above-mentioned organs. HUBBARD and BUTSCH (1941) declared that the sulfanilamide concentration in the bile most closely approached to that of the blood, without however becoming equal to it, being followed next by sulfapyridine and sulfathiazole.

An explanation of the differences in the concentration between the plasma, on the one hand, and the red blood corpuscles and the cerebrospinal fluid, on the other, is a more complicated matter. Apparently, the most widespread conception is that these differences are due to the varying degree of the binding of the sulfanilamide derivatives to the plasma proteins. Consequently, only part of the administered substance can be freely filtrated. This explanation was offered by ANDERSEN, MØLLER and SIMESEN (1942), as well as by DAVIS (1942).

DAVIS performed his investigation on the basis of dialysis experiments. According to him, as regards the unacetylated compound, 80 per cent of the sulfanilamide, 60 per cent of the sulfapyridine, 45 per cent of the sulfadiazine and 25 per cent of the sulfathiazole were free. MARSHALL et al. (1937) established that, at ultra-filtration under a pressure of 125 mm mercury, all the sulfanilamide could be freely filtrated. KIMMIG and WESELMANN (1941) found that serum retained sulfanilamide derivatives even at a pressure of 8 atmospheres.

Further, sulfapyridine is ultrafiltrated to a greater extent than sulfathiazole. They contended that pure adsorption occurred owing to the fact that the sulfanilamide derivatives could be shaken out with charcoal. In similar experiments, ANDERSEN, MØLLER and SIMESEN (1942) observed that 20—30 per cent of sulfathiazole can be filtrated when in the plasma. HERBERTS (1944) noted that the dialysable part of the sulfapyridine comprised 24—36 per cent of the concentration of the inner fluid. Also the concentration of proteins, and albumin, in particular, which binds the main part of the sulfanilamide preparations is of significance with regard to the extent of the filtrable fraction (REINHOLD, FLIPPIN, DOMM, POLLACH, 1944).

As regards the brain and the cerebrospinal fluid, they are, in addition, considered to be separated from the blood by a barrier. However, available knowledge regarding this barrier seems not to have been made use of in this connection.

No examinations regarding the exact localization of chemo-therapeutics in the tissues, i. e. their histo-pharmacology, have been noted, with the exception of HERBERTS' work referred to below. Nevertheless, a few methods have been described for ascertaining sulfanilamide derivatives in the tissues directly through a microscope. HACKMAN (1942) has given an account of a method using ALTMANN'S original freezing-drying method for the fixation of drugs. After cutting, the sections are examined through an ordinary microscope. The coloured chemo-therapeutics (prontosil solubile and rubrum) will then be visible owing to their strong red colour. The unstained preparations are diazotized in the sections according to the usual procedure. However, this method is comparatively unsensitive and large doses are required. In the published pictures, the doses are said to amount to 60 mg. This would imply a concentration of approximately 1: 300 with regard to a mouse of 20 g when a diffuse distribution is presupposed. HACKMANN'S work only describes the method and no results regarding the localization of the sulfanilamide derivatives in the tissues have been reported.

HERBERTS (1944) has described a similar method, employing instead ordinary frozen sections. He has only subjected the localization of sulfapyridine in the brain to examination, finding that this drug has an elective affinity to the ganglion cells.

Lastly, a method has been described by MAC KEE, HERMAN, BOOR and SULZBERGER (1943) as follows: The fixation is carried out in dry formaldehyde gas. Then, preferably, frozen sections are made, their thickness being, on an average, 16 μ . After this, Ehrlich's reagent and absolute alcohol acidified with HCl are dropped on to the sections. The sulfanilamide then becomes stained. However, the colour does not remain for more than 3—4 hours.

As regards the above-mentioned methods, the two latter ones do not fulfill the demands stipulated with regard to the fixation method in Part I, page 11. Nor do they answer to the requirements of the detection method on page 14. Furthermore, data regarding the sensitivity of the methods are lacking.

CHAPTER V

Own investigations regarding the localization of certain sulfanilamide derivatives to different tissue structures in various organs by means of fluorescence microscopy

Material

As pointed out earlier, the first question to be discussed is whether the azo linkage and the compound coupled with the sulfanilamide in this azo linkage are of any significance with regard to the localization of these drugs in the tissues. The drugs used for the analysis of this problem were prontosil soluble (Bayer) in a 2.5 per cent solution, sulfanilamide (Astra) in a pure substance, and sulfathiazole-Na (Astra) in a 20 per cent solution.

The examination was carried out on white mice, partly owing to the fact that these animals are most common in chemo-therapeutical animal experiments and, partly, on account of their particular suitability for the fixation method employed. Thus, when mice are used, the small organ sections will offer good survey pictures. Moreover, these small pieces are to be preferred, giving as they do the most adequate fixation by means of the freezing-drying method.

The largest material comprises animals treated with prontosil soluble which has been injected intravenously. The dose most often administered consisted of 0.5 ml of a 2.5 per cent solution. This particular dose was chosen, since it was stated by DOMAGK to be the lowest dosage required for the survival of all the animals subjected to streptococcal sepsis in experiments where the whole control material had died within the

first twenty-four hours. Other doses have been applied for special purposes. Injections directly into the cerebrospinal fluid have also been performed for studies of the relationship to the central nervous system.

As regards the sulfanilamide and the sulfathiazole, the material adopted for these preparations is less comprehensive than in the case of prontosil soluble, for reasons which will appear further on. Different doses have been given at intramuscular or intravenous injection. The intramuscular injection has been resorted to for the supply of larger doses during longer periods of time. Since sex has been found not to be of any significance with regard to the general distribution in the examined organs and tissues, this has not been specified. Nor have the weights been set down. They have varied from 17 to 22 g. The animals have been killed by cutting off the head.

The distribution of prontosil soluble in the tissues at different periods after intravenous injection

**15 sec.—1 min. after 0.2 and 0.5 ml 2.5 % sol.
(7 animals).**

The auto-fluorescence of the tissues appears, as mentioned earlier, chiefly in different shades of blue and grey. In addition, yellow fluorescent granula occur. No red fluorescence has been observed. Nor has this been described by F. SJÖSTRAND (1944) who gives close details of the auto-fluorescence of tissues, as well as an exhaustive account of the observations made by earlier authors in this field. Considering that all the red fluorescence detectable in the tissues (which is possible without heating the drugs) must be attributed to the presence of prontosil, no special justification has been regarded as necessary for stating simply, in the description of the distribution in the various tissues, that prontosil occurs wherever the red fluorescence colour is observed. On the other hand, it cannot, of course, be definitely concluded that a tissue is free from prontosil on account of the absence of the red fluorescence. The

prontosil may, very likely, occur in the tissues without being detectable, since its concentration lies below the one ascertainable by this method. This reservation must always be kept in mind when prontosil is said to be lacking in a tissue or organ.

In the description of the organs, the central nervous system has been excluded owing to the specific conditions occurring there. These circumstances have been dealt with in Chapter VI.

The connective tissue. The connective tissue, which has an auto-fluorescence in different shades of blue, contains large quantities of prontosil in these test animals, easily discernible by its red fluorescence. This red fluorescence proceeds from certain coarse and fine fibrils, the position of which is found to correspond to that of the collagenous and elastic fibrils when the slices are stained according to van Gieson's and Unna's orcëin staining methods. (When, in the following, the drugs are said to be localized to certain tissue structures, cells, etc., this localization has been further controlled by staining the sections with ordinary histological dyes. As a rule, hematoxylin-eosin, van Gieson, Unna's orcëin, as well as the Weigert elastin and Mallory connective tissue staining methods have been used.) The blue-white fluorescence of the cells in the connective tissue contrasts strongly with the red colour of the prontosil. The fluorescence picture of the connective tissue is, on the whole, identical wherever it occurs. Attention should be directed to the fact that the distribution of the red colour is dependent on the occurrence of elastin and collagen.

Adipose tissue. This tissue has retained its blue-white fluorescence and reveals no red fluorescence colour.

Skeletal muscle. The muscle bundles have entirely kept their blue auto-fluorescence, as well as the yellow-grey in isolated muscle bundles. The intervening connective tissue contains prontosil. The nervous fibers in the musculature retain their intense blue-white fluorescence.

Tendons. The tendons have a marked red fluorescence.

Smooth musculature. These muscles have approximately the same auto-fluorescence as the striated musculature and are quite free from red fluorescence.

Cardiac muscle. The cardiac muscle has also retained its blue-white auto-fluorescence and does not contain any visible red substance. On the other hand, the elastin and collagen lying between the muscular bundles is red. The endothelial cells of the endocardium also have a red fluorescence.

Vessels. In the aorta, the elastin which normally has an intensely pale blue auto-fluorescence (Fig. 4 b) is strongly red in the animals subjected to prontosil treatment, while the intervening smooth muscle bundles have kept their blue colour. (Fig. 4 c.) The border lines of the elastin are very sharply defined. No trace of the red colour is visible between these membranes. As regards the small arteries of, e. g., the kidney, only the internal elastic membrane and the elastin and collagen of the adventitia are stained (Fig. 7).

Lung. The endothelium in the small vessels is red. Fine red fibrils are observed in the alveolar walls, corresponding to the elastin. The epithelium in the bronchioli and bronchi has completely retained its blue-white fluorescence colour.

Spleen. At low magnification (obj. 20 \times), the splenic capsule and trabeculae have a pure red appearance. Only when the magnification has been increased (obj. 42 \times), is the red colour found to be arranged in a very fine network, corresponding to the appearance of the elastic and collagenous fibers. The lymphatic tissue is quite free from prontosil, as well as the red pulp which, however, is much darker than the white pulp owing to its abundant amount of red blood corpuscles. The cells containing granules fluorescing in yellow, which have been proved referable to the R. E. S. by F. SJÖSTRAND (1944), are free from prontosil.

The lymphatic glands. Broadly speaking, the distribution of prontosil is the same as in the white pulp of the spleen. No red fluorescence is found in the sinus endothelium and the reticular cells.

Liver. Apart from the vessels which are red, in accordance with an earlier description, a large quantity of the finest bile capillaries contain prontosil already after 15 seconds. After one or two minutes, also the bile ducts are red. The cells



Fig. 4 a. Aorta of mouse, killed 80 min. after i. v. inj. of 0.5 ml 2.5 % sol. of Prep. I. Elastic membranes brown.

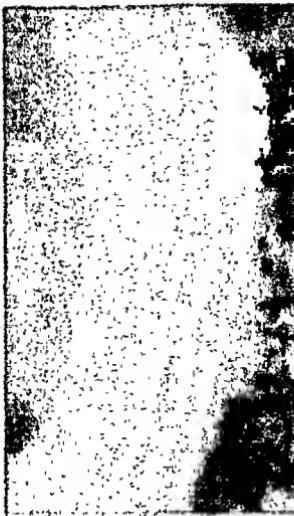


Fig. 4 b. Aorta of untreated mouse. Elastic membranes pale blue.



Fig. 4 c. Aorta of mouse, killed 1 min. after i. v. inj. of 0.5 ml 2.5 % sol. of prontosil soluble. Elastic membranes red.

Obj. Imm. (90 x). Exposure 30 min.



Fig. 5. Skin of mouse, killed 1 min. after i. v. inj. of 0.5 ml 2.5 % sol. of prontosil soluble.

Obj. 42 x. Exposure 30 min.

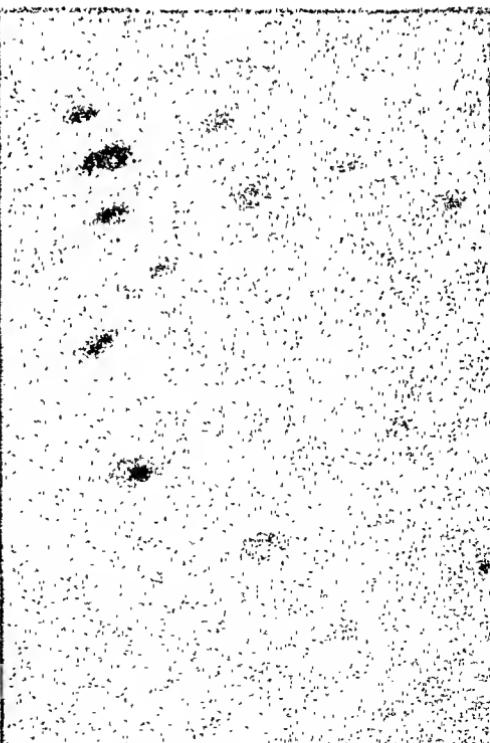


Fig. 6. Brain of mouse, killed 35 min. after inj. in cerebrospinal fluid of 0.1 ml 2.5 % sol. of prontosil soluble.

Obj. Imm. (90 x). Exposure 30 min.

of the liver have not lost their blue fluorescence. The same applies to the dark blue colour of the cellular nuclei. The stellate cells of v. KUPFFER have kept their auto-fluorescent property and lack all traces of red fluorescence. Their yellow granula emit light equal in intensity to that of the untreated animals.

Gall bladder. In the gall bladder, the epithelium is blue-white, while the submucous connective tissue is red. In the lumen no contents are, as a rule, detected. However, now and then an intensely deep green fluorescent substance is observed as in the untreated animals.

Stomach. All the epithelial cells fluoresce in blue. No prontosil is found in the lumina. Similarly to the connective tissue in the submucosa, the interstitial connective tissue between the glands is red.

Intestines. The epithelial cells are a pure blue. In the connective tissue between the glands a red fluorescent substance is visible. Prontosil soluble is found only in the proximal part of the intestinal lumen. The connective tissue in the submucosa is stained a strong red colour.

Kidney. Animals killed 15 seconds after the injection. The glomeruli were strongly fluorescent in red. In H₁* a high prontosil concentration occurred in the brush border. Prontosil is found also in the lumen, with, however, a less high concentration than in the brush border. In H₂, H₃ and H₄ and in a distal direction, prontosil is lacking both in the lumen and intracellularly.

30 seconds after the injection. The glomeruli are somewhat paler than in the preceding specimen, but evidently still contain much prontosil. Prontosil soluble is found in the whole of the proximal convoluted tubule in the brush border. Distally to the proximal convoluted tubule, the tubules are free from prontosil (Fig. 9).

One minute after the injection. The glomeruli contain somewhat less prontosil than in the preceding speci-

* [The classification of the proximal convoluted tubules as H₁, H₂, H₃ and H₄ has been made in accordance with F. SÖÖSTRAND (1944).]

mens. The same applies to H_1 which is faintly red in the brush border. In H_2 the apical cellular zones have a high prontosil concentration. H_3 and H_4 reveal a stronger red fluorescence in the apical part of the cells than H_1 , but are weaker than in H_2 . The brush border in H_3 and H_4 has a more marked red fluorescence than in the preceding specimens. Distally to the proximal convoluted tubule, prontosil soluble occurs only in the lumen, i. e. not intracellularly. In the ascending limb of Henle's loop and distally, a considerably higher concentration in the lumina than proximally is noted (Fig. 7).

Bladder. The epithelium is a pale blue-white. The submucous connective tissue is red in accordance with an earlier description. The animals, which were killed 1 minute after the injection, already had prontosil in the lumen of the bladder.

Skin. The horny layer and the epithelium in the epidermis have kept their blue-white fluorescence. The muscle bundles are bluish or yellowish. All the glandular cells (sebaceous glands, sweat glands) and the hair follicles, as well as the hairs, have retained their blue auto-fluorescence. The nervous fibers are intensely blue-white. In the connective tissue the collagenous bundles and elastic fibers are stained. Accordingly, only the cellular elements of this elastin- and collagen-rich connective tissue have a blue-white colour (Fig. 5).

Ear. The elastic cartilage in the ear, which normally has a blue fluorescence, is red in the animals treated with prontosil.

15 min. after 0.2 and 0.5 ml 0.25—2.5 % sol.,

0.5 ml 2.5 % sol. (5 animals).

Connective tissue, adipose tissue, skeletal muscle, smooth muscle, vessels, heart, lung, spleen, stomach, skin and ear, disclose the same picture as described earlier.

Liver. Practically all the bile capillaries and bile ducts are red. The liver cells and the v. KUPFFER cells are free from red fluorescence.

Gall bladder. Filled with red substance. In several places, the cytoplasma of the epithelial cells and the cellular nuclei

are red, the latter having a darker red colour. The submucous connective tissue has the same appearance as earlier.

Intestines. In the lumen of the proximal parts of the small intestine, an abundance of intensely red prontosil soluble. The epithelium, as well as the glands, as a rule, retained its blue auto-fluorescence. As previously, prontosil soluble occurs in the connective tissue of the villi. A pronouncedly red colour is manifest in the mucosal connective tissue. The appearance of the most distal intestinal sections entirely conforms with the specimens described earlier.

Kidney. The glomeruli have a very insignificant red fluorescence. The proximal convoluted tubules have a scarcely noticeable red fluorescence in the apical cellular zone. A moderately diffuse staining of the apical cellular parts is noted in the distal part of the ascending limbs of Henle's loop and in the distal convoluted tubules. A fairly strong red fluorescence occurs in the lumina. In the collecting tubules, an intense red staining of the whole cell was observed. In some cases, a fainter red fluorescence has been found in the basal zones of these tubules, similar to the distal convoluted ones.

Bladder. The lumen is full of prontosil. *In spite of the high concentration, the epithelium is quite free from red fluorescence.*

Eye. In the cornea, the epithelium has a blue fluorescence, while the substantia propria is strongly red in colour. Also the endothelium has a red fluorescence. The epithelium in the ciliary processes has a blue auto-fluorescence, but the connective tissue is faintly red. The epithelial cells of the iris are blue, while the thin connective tissue is red. The retina lacks red fluorescence. The same applies to the vitreous body. The connective tissue of the sclera is pronouncedly red.

0.2 ml 2.5 % sol. (i. e. 5 mg, 2 animals).

The localization of the prontosil is the same as described above. The colour is possibly, a somewhat paler red. Further, in some parts as, for instance, in the connective tissue of the

intestine the colour is hardly visible. In *aorta, skin, liver, gall bladder, kidneys* and *bladder*, the difference in the colour is not particularly marked.

0.5 ml 0.25 % sol. (i. e. 1.25 mg, 2 animals).

Prontosil is ascertainable after 15 minutes only in the collagenous and elastic fibers (e. g., in the *aorta*) and in the *gall bladder*, the *bladder* and the *intestine*, where it is found at fairly high concentrations.

1 hour after 0.5 ml 2.5 % sol. (5 animals).

Connective tissue. In the connective tissue lacking in collagen and elastin as, for instance, in the interstitial connective tissue between the glands of the stomach, scarcely visible quantities of prontosil now occur. However, in a connective tissue which is richer in elastic and collagenous fibers, e. g., the skin, the picture is almost unchanged.

Adipose tissue, skeletal muscle, smooth muscle, cardiac muscle, vessels, skin, ear, eye, gall bladder and bladder showed unchanged pictures.

Lungs. The lungs have in these specimens, a very faint red colour in the elastic network.

Spleen. The spleen discloses a red fluorescence mainly in the capsule and very sparsely in the trabeculae. There is still no prontosil in the cells.

Lymphatic glands. The lymphatic glands have no visible prontosil.

Liver. The red fluorescence remains only in the coarser bile ducts.

Stomach. Prontosil only remains in the submucous connective tissue. The lumen is still free.

Intestines. Prontosil is found in large quantities in the lumen, as well as down in most of the crypts and glandular lumina. Prontosil also occurs in the interstitial and submucous

connective tissue, as in the case of earlier specimens, though with a fainter red fluorescence colour. The drug also appears down in the most distal intestinal parts of the large intestine and the appendix. The epithelial cells are free from red fluorescence.

Kidney. Prontosil has been ascertainable in some animals in one or two collecting tubules. Otherwise prontosil now only occurs in the elastin and collagen, e. g., in the vessels.

4 hours after 0.5 ml 2.5 % sol. (5 animals).

The red fluorescence is now only visible in the *gall bladder*, which still looks the same as in the above description, the *urinary bladder*, where prontosil occurs in the lumen, and in the *intestines*. In the latter organ, prontosil is found mostly in the lumen of the distal parts of the intestines. The connective tissue of the villi and of the submucosa are, as a rule, free from prontosil.

24 hours after 0.5 ml 2.5 % sol. (5 animals).

Prontosil cannot be detected anywhere.

**The distribution of prontosil soluble in the tissues 12—24 hours after repeated doses
(6 animals)**

In order to examine whether any prontosil has been taken up into the tissues, e. g., in the R. E. S., as in the case of many vital dyes such as trypan blue, varying doses have been administered several times daily for periods of up to 7 days. After the subcutaneous injection of 0.5 ml of a 2.5 per cent solution three times daily for 7 days, no traces of prontosil were detected in the tissues when the animals had been killed 12 and 24 hours, respectively, after the last injection. *Thus, no storing has been observable in the R. E. S.*

**The distribution of prontosil soluble in the skin after
local application
(6 animals)**

Prontosil soluble has also been applied in a 5 per cent ointment on the skin (adeps lanae being used as a vehicle). The treated surface was covered. At different times after the application, i. e. 5 minutes, 1 hour, 2 hours, and 24 hours, skin pieces were removed. Prontosil was not ascertainable in any single case, except for the most superficial part of the horny layer. This finding was obtained irrespective of whether the ointment was given several times during the course of a couple of hours and rubbed into the skin each time for a few minutes.

**The distribution of sulfanilamide and sulfathiazole
in the tissues at different periods after
intravenous injection**

Both these drugs can be detected in the tissues after heating to 160° for 5 min. (sulfanilamide) and 175° for 5 min. (sulfathiazole). Thus, they become yellow while the tissue is blue. After the administration of varying doses up to 0.5 ml of a 5 per cent solution intramuscularly and 0.5 ml of a 2.5 per cent solution intravenously one hour later, these drugs have been ascertainable only in the kidneys and in the bladder and, at intramuscular injection, in the musculature round the injection spot.

**Sulfanilamide after 0.2 and 0.5 ml 2.5 % sol.
(15 animals).**

5 min. — 1 hour after the injection.

Kidney. In the lumina of H₃ and H₄ which are dilated, a substance fluorescing in yellow is noticed. It also occurs in all the lumina distally to the proximal convoluted tubules. In the lumina of the collecting tubules a stronger yellow-brown fluorescence is found than in the tubular sections. No sulfa-



Fig. 7. Kidney of mouse, killed 1 min.
after i. v. inj. of 0.5 ml 2.5 % sol. of prontosil soluble.
Obj. 42 \times . Exposure 45 min.



Fig. 8. Kidney of mouse, killed 1 hour after
i. v. inj. of 0.5 ml 1.25 % sol. of sulfathiazole-
Na. Section heated to 175° for 5 min.
Obj. 1mm. (90 \times). Exposure 15 min.



Fig. 9. Kidney of mouse, killed 30 sec. after i. v. inj. of 0.2 ml 2.5 %
sol. of prontosil soluble.
Obj. 42 \times . Exposure 45 min.



nilamide was ascertainable intracellularly. The internal elastic membrane of the small arteries reveals no change of its usual pale blue auto-fluorescence.

Bladder. A large amount of substance fluorescent in yellow occurs in the lumen of the bladder. The bladder epithelium has retained its auto-fluorescence.

In the remaining organs which have been subjected to examination (similar to the prontosil animals), no change has been observable in the auto-fluorescence.

Sulfathiazole after 0.2 ml 2 % sol.

5 minutes after the injection. (5 animals)

Kidney. A substance fluorescent in yellow is detectable in the lumina of the collecting tubules. Otherwise the cells, connective tissue and vessels of the kidneys have completely retained their usual pale blue auto-fluorescence after heating to 175° for 5 min.

Bladder. Sulfathiazole is detectable in the bladder lumen.

15 minutes after the injection (5 animals).

Kidney and bladder. The same appearance as in the former specimen. Sulfathiazole is detectable in the collecting tubules and the bladder already after a dose of 0.5 ml of a 0.1 per cent solution given intramuscularly.

1 hour after the injection (5 animals).

Kidney. The glomeruli disclose an unchanged auto-fluorescence. The proximal convoluted tubules are unstained in the lumen as well as in the cells. The ascending limb of Henle's loop, the distal convoluted tubules, and the collecting tubules have a strong yellow fluorescence and contain sulfathiazole at a high concentration in the lumen as well as in the cytoplasma. The cellular nuclei, on the other hand, are unstained. The in-

ternal elastic membrane of the small arteries has completely kept its auto-fluorescence (Fig. 8).

Bladder. A large amount of substance fluorescent in yellow occurs in the bladder lumen. The epithelium has kept its auto-fluorescence.

Other organs and tissues have not revealed any change in the auto-fluorescence. Accordingly, they have not been subjected to a detailed examination.

Survey of the results

The distinctive feature of prontosil soluble is its affinity to elastin and collagen. Consequently, prontosil soluble may be defined as a vital dye with regard to these structures, which are pronouncedly stained already 15 seconds after an intravenous injection of as small quantities as 1 mg/20 g of body weight. The colour remains in the elastic and collagenous fibers even after the disappearance of the prontosil from most of the other organs and only slight amounts are detectable in the liver and the kidneys where the prontosil is excreted. In the gall bladder, the urinary bladder and the intestinal canal, prontosil is still ascertainable 4 hours after the administration of a dose of 12.5 mg (as regards the choice of dose, see further page 53). Evidently, some of the prontosil dose circulates from the liver via the bile ducts to the intestine and is then resorbed. It is, thereupon, transported on the blood pathways to the liver and excreted again into the intestine. *Thus, also an intravenous supply of prontosil will give high concentrations in the intestinal lumen.*

Further, the low concentrations generally occurring in the epithelium are noteworthy. In several instances, they fall below the limit of sensitivity of the method employed. Consequently, their existence in the cells cannot always be definitely established. In other cases, it may be concluded that they must probably have wandered through the cells as, for instance, in those of the liver. The other epithelial cells (among those examined) which have contained prontosil soluble in detect-

able concentrations are the gall bladder epithelium, and the epithelial cells in some parts of the renal tubules.

And yet, the epithelium has been in direct contact with the prontosil at high concentrations in several other parts, such as the bladder, the glandular lumina of the intestines, the glandular epithelium and the superficial epithelium of the skin, etc. This may, conceivably, be due, partly, to the penetration of prontosil at very low concentrations, as, e. g., through the hepatic cells, partly, to the impermeability of such cells as, for instance, the bladder epithelium.

Further, the musculature, adipose tissue, peripheral nerves, and (as will be derived from Chapter VI) the central nervous system do not contain detectable quantities of prontosil. Nor has any storing been observable in the R. E. S.

Accordingly, prontosil solubile has an exceedingly uneven distribution in the organism from the very moment it is injected. This uneven distribution is not equalized until all the concentrations begin to approach to the limit value of detectability. The high concentrations obtained with prontosil occur in the connective tissue and, above all, in the connective tissue rich in collagen and elastin, in the bile ducts, in some parts of the kidneys, and in the intestines.

Sulfanilamide and sulfathiazole lack an elastin and collagen affinity. They are not manifest in these structures even at high doses, being observable only in the kidneys, the ureter and the bladder. Since the method is not sensitive enough for the detection of these drugs in concentrations equal to those of the plasma, these drugs cannot be expected to occur in the elastin and collagen, provided they do not possess an affinity to these substances resembling that of the prontosil solubile.

If such an affinity existed and the drugs appeared in concentrations corresponding to those of the prontosil, they should, in all probability, be ascertainable there. For prontosil solubile, as well as all the other examined substances with an elastin affinity (see further Chapter VIII), are detectable without any difficulty, for instance, in the internal elastic membrane of the renal vessels, even though only very small quan-

ties of these substances remain in the tubular cells or the lumen. On the other hand, no staining of this internal elastic membrane has, in fact, been noticeable with sulfanilamide and sulfathiazole, even when large amounts have been found in the tubular cells and the lumina (Fig. 7 and 8).

The fact that prontosil has an elastin and collagen affinity, while sulfanilamide and sulfathiazole lack this property, can also be demonstrated supravitally. Thus, when tissues containing elastin and collagen are placed in solutions of these three drugs, prontosil will be found to have coloured the elastin and collagen a faint red already at a concentration of 1: 50 000, while sulfathiazole and sulfanilamide do not stain the elastin and collagen until a concentration of about 1: 1 000 is reached, which causes the surrounding tissues (muscles, etc.,) to become equally strongly coloured.

The fact that prontosil does not penetrate intact skin, or does not, at any rate, do so in concentrations to such a degree as to be detectable by means of this method, is in agreement with MIESCHER'S (1941) finding with salicylic acid, trypanflavin and other dyes, such as eosin, rhodamin, etc. These substances did not penetrate the epidermis in ascertainable concentrations after application to the ear of a guinea-pig, irrespective of the vehicle employed. *As regards prontosil, the concentration obtained by means of intravenous injection is higher, beyond comparison, than that of local application, when the skin is intact.*

Summary

By means of fluorescence microscopy, prontosil has been proved to have an affinity to elastin and collagen, while sulfanilamide and sulfathiazole lack this affinity. It this way, high concentrations of prontosil are attained in the connective tissue and, above all, in the one rich in elastin and collagen. Thus, the highest concentration is met with, inter alia, in the skin, the lungs, the tendons, the ligaments, and the elastic cartilage. High concentrations also occur in the organs where the pron-

tosil is excreted. However, it is often not even detectable intracellularly in these organs, but rather in the lumina, e. g., the bile capillaries, the bile ducts, the intestinal lumina, the renal tubules and the bladder. Prontosil is seen intracellularly in the epithelium of the gall bladder and in some parts of the tubules.

The collagenous and elastic fibers act as a depot for prontosil which remains there for a longer time than in any other part of the organism with the exception of the bladder, the gall bladder, and the intestinal lumen.

No storing occurs in the R. E. S.

Prontosil does not penetrate intact skin in detectable concentrations, at least not when applied in the form of an ointment.

Sulfanilamide is only detectable in the lumina of the renal tubules, the excretory passages of the kidney, and in the bladder lumen.

Sulfathiazole can be observed in the renal tubules, in the lumina, as well as intracellularly in the distal tubules and in the bladder.

CHAPTER VI

Investigations regarding the localization and passage of sulfanilamide derivatives to the central nervous system

Introduction

As already derived from Chapter IV, the various sulfanilamide derivatives occur in different concentrations in the brain and in the cerebrospinal fluid. Sulfanilamide is the compound with a concentration in the cerebrospinal fluid approximating the most to the blood concentration, being about 60—100 per cent. Sulfapyridine comes next with about 40—90 per cent, and is followed by sulfathiazole with 10—40 per cent. Prontosil soluble and rubrum do not, apparently, penetrate into the cerebrospinal fluid.

This may be explained in several ways, as reported below.

1. On the basis of dialysis experiments, it has been found that the sulfanilamide derivatives are combined with the *plasma proteins* and that, in consequence, only part of them can be freely filtrated. According to these results, about 80 per cent of the unacetylated sulfanilamide are free, 60 per cent of the sulfapyridine, and 25 per cent of the sulfathiazole. These results have been confirmed by several investigators (cp. Chapter IV).

According to the literature dealing with the subject of the blood-brain and the blood-cerebrospinal barriers, also the following factors are of significance.

2. *The solubility of the substances.* Avertin, for instance, is more soluble in tissues rich in lipoids, such as the brain, than in the plasma.

3. *The electrical charge of the substances.* This is a factor of decisive importance. Positively charged substances easily penetrate into the cerebrospinal fluid, while the negative ones do so with difficulty, and less in proportion to their increasing negativity.

4. *The degree of dispersion.* Particularly big molecules cannot pass through the barrier..

The rôle played by the two last-mentioned factors have not, as far as I have been in a position to establish, been subjected to a more detailed examination regarding the sulfanilamide derivatives. Prior to the demonstration of a binding to the plasma proteins, it was considered sufficient to refer merely to the existence of a barrier. Since then, the plasma-protein explanation, seems to have been accepted without reservation.

Own investigations

1—60 min. after the intravenous injection of 0.5—1.0 ml of a 2.5 % sol. of prontosil soluble.

The material consists of all the animals in Chapter V treated in this way. The brain was found to be colourless in the macroscopical examination. Prontosil soluble was only detectable microscopically in the vessels and vascular walls of the brain and the spinal cord, even after the intravenous supply of doses as large as 24 mg. It was discovered in the endothelium of the cerebral vessels, as well as in the meningeal vessels.

10—60 min. after the injection in the cerebrospinal fluid of 0.05—0.1 ml of a 0.25—2.5 % sol. of prontosil soluble (10 animals).

Prontosil had a marked affinity to the ganglion cells after the injection in the cerebrospinal fluid, several of the cells being intensely coloured. The nuclei as well as the nucleoli were stained (Fig. 6).

The remaining cerebral substance still had its usual auto-fluorescence. A narrow space is often seen round the vessels

visible in the cerebral substance. This space often contains a substance fluorescing in red.

When, on the other hand, a piece of the brain is removed and dipped into a prontosil solution of a similar concentration, quite a different picture will appear. The cerebral substance is diffusely stained, its colour diminishing in intensity from the surface inwards, with a vague limit where the diffusion ceased owing to the fixation. This limit lies at different depths, according to the length of time during which the brain was soaked in the dye solution. However, also in this case the ganglion cells appear more strongly coloured than the rest of the cerebral substance, with the exception that at this time all the cells are stained.

After peroral administration (cp. page 84) of prontosil rubrum (8 animals).

A macroscopically visible yellow colour of the brain was observed. No accumulation was ascertainable in the ganglion cells by means of the microscope.

3—60 min. after injection in the cerebrospinal fluid of 0.05 ml of a 1 % sol. of prontosil rubrum (8 animals).

The cerebral substance round the injection spot was found to be markedly stained by prontosil. However, the staining did not extend over a particularly wide region, including only a very superficial layer of the brain substance. The colour remained unchanged 1 hour after the injection.

In the microscope, the superficial layer of the grey substance appeared diffusely stained with prontosil. The ganglion cells did not have a stronger colour than their surroundings.

After the intravenous injection of 0.5 ml of a 2.5 % sol. of chrysoidine (5 animals).

A macroscopically visible yellow staining of the brain was noted. In animals where the brain had been removed after 15 minutes, the colour was very pronounced and remained quite

distinct even in animals killed 2 hours after the injection. The colour had vanished in animals killed after 4 hours. The animals were unaffected. No specific localization in the ganglion cells was detectable microscopically. The cerebral substance seemed of a fairly even yellow colour. Nor was the chrysoidine visible in the ganglion cells after an injection into the cerebrospinal fluid. (3 animals.)

15—60 min. after the injection of sulfanilamide, sulfathiazole and sulfapyridine intravenously as well as in the cerebrospinal fluid.

Sulfanilamide, sulfathiazole and sulfapyridine have not been observed in the brain after intravenous injection (as regards the material, cp. page 62). Nor have these drugs been noted in the ganglion cells after injection in the cerebrospinal fluid, (doses 0.05—0.1 ml 2 % sol. 5 animals to each drug). Accordingly, no support can be found for the assumption of an elective localization of these drugs in the ganglion cells. Thus, it has not by means of this method been possible to verify HERBERT'S results concerning this problem.

Discussion of the results

The question is, do these findings conform with our present knowledge of the blood-brain barrier. The following facts have been derived from BROMAN'S (1940—41) investigations which constitute, at present, the most comprehensive modern surveys in this field.

A distinction should be made between the blood-cerebrospinal barrier, on the one hand, and the blood-brain barrier, on the other. The occurrence of a blood-brain barrier is considered definitely proved. It may be described, as follows: 1) It is, in all probability, localized to the vascular intima of the cerebral vessels. 2) This membrane admits the penetration of basic substances only, such as dyes, toxins, drugs, etc., and not acid ones. FRIEDEMANN (1937), who propounded the theory that the blood-brain barrier consists of an electro-ne-

gatively charged membrane, has shown by experiments with toxins that this passage is dependent not only on the particular kind but also on the degree of the electrical charge. Thus, the hindrance to passage increased in proportion to the electro-negative charge. As compared with this, the degree of dispersion and solubility are factors of subordinate importance, except in unusually pronounced cases. Finally, as already mentioned, the binding to the plasma-proteins also plays a certain part with regard to the concentration in the cerebrospinal fluid.

On the other hand, the existence of a blood-cerebrospinal barrier has not been generally acknowledged. This barrier may, conceivably, from an anatomical point of view, be localized to the vessels of the meninges and to the chorioid plexus. To all appearances, BROMAN considers that a blood-cerebrospinal barrier, which acts similarly to the blood-brain barrier, does not exist, in spite of the hindrance to the passage of acid dyes. The fact that basic dyes are, as a rule, unascertainable in the cerebrospinal fluid though they permeate the blood-brain barrier and stain the cerebral substance, is explained by BROMAN as due to their adsorption to this substance. He states that the difference between the chorioid plexus and other vessels lies in the marked staining of the whole of the cells of the chorioid plexus. On the other hand, the vessels of the meninges resemble more the cerebral ones, where only the endothelium is stained. However, the chorioid plexus is pointed out as, in many respects, resembling a gland with, possibly, a secretory function. Contrary to BROMAN, it may be contended that the chorioid plexus may, from a functional point of view, constitute a similar barrier to that of the cerebral and meningeal vessels (i. e. they are permeable to a varying degree with regard to different substances), in spite of the deviating histological structure, as compared with these vessels. Nor, in fact, does the total staining of the chorioid plexus exclude the possibility of such a function.

My experiments seem to confirm the conception that a blood-brain barrier, as well as a blood-cerebrospinal barrier,

exist with, on the whole, the same type of function. The decisive factor with regard to the permeability is the electrical charge of the substances. Prontosil soluble which is negatively charged does not pass through, while chrysoidine which is positively charged, and prontosil rubrum, which is, probably, only slightly acid, penetrate. The, in all probability, weaker acidity of prontosil rubrum than of prontosil soluble is evident from their formulae. Prontosil rubrum only contains one acid group, i. e. the faintly acid sulfonamide group. Prontosil soluble, on the other hand, contains 2 sulfonic-acid groups, the acidity of which is very marked. Further, it is well known that the purely basic chrysoidine, as well as the pure sulfanilamide, penetrate the barrier and attain fairly high concentrations in the central nervous system. Since the sulfonamide group is not sufficiently acid to prevent the passage of sulfanilamide, it is not, *a priori*, likely that this group will stop the passage of the substance formed, viz. prontosil rubrum, when substituting one of the amino groups of the chrysoidine. The fact that prontosil rubrum is unascertainable in the cerebrospinal fluid in spite of its penetration of the barrier is probably due to its being taken up in the cerebral substance, similar to chrysoidine and other basic dyes. These experiments are in agreement with BROMAN'S in this respect.

Since prontosil soluble is not detectable in the cerebrospinal fluid, nor in the ganglion cells, after an intravenous injection, the existence merely of a blood-brain barrier will not suffice as an explanation of the above-mentioned facts. Thus, a barrier must also occur between the blood and the cerebrospinal fluid. For prontosil soluble is, obviously, detectable in the ganglion cells when present in the cerebrospinal fluid. In this respect, the effect of prontosil resembles that described by GOLDMANN (1913) in his classical experiments with trypan blue. He also discovered that after a subarachnoidal injection of trypan blue in rabbits, the ganglion cells appeared to a large extent to be diffusely blue in colour, the nucleus in the ganglion cell invariably being stained. These coloured cells were detectable even though the brain was unstained at the macroscopic examination.

tion. GOLDMANN explains this phenomenon as due to the existence of a direct communication between the cerebrospinal fluid and the ganglion cells via the perivascular lymphatic spaces first described by KEY and RETZIUS (1875). No better explanation of how prontosil and trypan blue can reach the ganglion cells has been offered.

As regards unstained sulfanilamide derivatives, a distinct correlation is found when the concentration of these drugs in the cerebrospinal fluid is viewed in relation to their dissociation constants. Data regarding the dissociation constants have been offered by BELL and ROBLIN (1942) and are set down in Table 4. As will be seen, the least acid substance, i. e. sulfanilamide, has the highest concentration in the cerebrospinal fluid, while the most acid one, i. e. sulfathiazole, has the lowest concentration. Sulfapyridine lies in between the two.

Table 4. *The relationship between acid constants and concentrations in cerebrospinal fluid of various chemotherapeutics.*

Compound	Acid constants		$K_b \times 10^{12}$		Conc. in cerebrospinal fluid in % of blood conc.
	pKa	Ka	1st	2nd	
Sulfanilamide . . .	10.43	3.7×10^{-11}	2.3	—	60—100
Sulfapyridine . . .	8.43	3.7×10^{-9}	3.8	0.1	40—90
Sulfathiazole . . .	7.12	7.6×10^{-8}	2.3	—	10—40

These results conform well with available data regarding the blood-brain and blood-cerebrospinal barrier. Nevertheless, in what way do the observations of the part played by the plasma proteins agree with these findings? Apparently, the varying cerebrospinal concentrations of unstained chemo-therapeutics have been adequately accounted for also by this binding. Thus, two explanations offer themselves in this respect, each being, obviously, satisfactory *per se*.

The question arises whether some kind of connection exists between them. From a study of the literature (cp. BROMAN) it will be seen that the assumption that the barrier between the

blood, on the one hand, and the brain and the cerebrospinal fluid, on the other, is a negatively charged membrane, is based, partly on the well confirmed observation that basic dyes are capable of vitally staining the central nervous system, in contrast to the acid ones, and, partly, on FRIEDEMANN'S experiments with toxins which have been mentioned above. This applies to normal material, while under pathological conditions the barrier does not function to a full extent, but can be penetrated also by acid dyes, proteins, etc. However, in these experiments, the possibility of a binding to the plasma proteins has not been examined. It is, therefore, conceivable, that in these cases, too, the binding with the plasma proteins plays an important part with regard to the amount which may pass into the cerebrospinal fluid and the brain. It seems possible that the phenomenon which has been described as a barrier function is, actually, a function of the binding to the plasma proteins. Thus, the degree of this binding may be dependent on the degree of acidity of the substances.

Against this hypothesis may be quoted the fact that, if a binding to the plasma proteins is the predominant factor, the non-existence of, for instance, prontosil soluble in the cerebrospinal fluid at a concentration corresponding to the freely filterable fractions is difficult to explain. In this respect, it should be borne in mind that prontosil soluble has a molecular weight below that of substances which are transmitted to the central nervous system and is also easily diffusible.

In ultrafiltration tests, a certain amount of the prontosil in the plasma has been found liable to free filtration (see Table 5). This was suspected already on account of the results obtained from electrophoresis experiments (cp. page 85), since one prontosil fraction wandered more rapidly than the plasma proteins.

Chrysoidine, on the other hand, which can be ultrafiltrated to approximately the same extent, stains the central nervous system. Sulfathiazole, which can be ultrafiltrated to but a slightly greater extent, also occurs in the brain and in the cerebrospinal fluid at concentrations equalling about 10—40 per

Table 5. *The ultrafiltrability of some chemo-therapeutics and chrysoidine.*

Compound	Plasma No.	Conc. in plasma before ultrafiltration	Conc. in the proteinfree ultra-filtrate	% ultra-filtrated substance	Protein conc. in plasma	
					% Total	% Albumin
Sulfanilamide	I	10.30	8.28	80		
	II	9.06	7.27	80		
	III	10.00	7.33	73		
Sulfapyridine	I	9.13	3.39	37		
	II	9.77	3.70	38		
	III	10.00	3.10	31		
Sulfathiazole	I	10.00	1.73	17		
	II	10.00	3.18	32		
	III	9.55	1.70	18		
	IV	8.86	1.77	20		
Prontosil sol.	III	10.00	1.50	15		
	V	25.40	2.50	10		
	VI	9.00	1.15	13	7.85	3.95
	VII	20.00	2.30	12	7.70	5.04
	VIII	17.80	2.80	16	7.60	4.68
	IX	14.80	3.45	23	8.05	5.02
Chrysoidine	V	15.00	1.20	8		
	X	28.00	6.10	22	6.65	4.35
	XI	21.10	1.75	8		

cent of that of the plasma. In my opinion, this seems inexplicable if the barrier function is considered only to be maintained by the plasma proteins.

Method of ultrafiltration. An apparatus described by P. LAVIETES (1937) has been employed for the ultrafiltration. This apparatus consists, broadly speaking, of two flattened semi-spherical glass chambers with flat sides. On one of these sides, the surface of which is ground very smooth, a large hole occurs. The two glass chambers are pressed against one another so as to place the plane surfaces in contact. The membrane is clamped between them. Each chamber communicates by means of a rubber tube with a levelling bulb containing mercury. The filtration pressure may be regulated by varying the level between the two bulbs.

In my experiments, a cellophane membrane has been used. This membrane is suitable owing to the fact that it is almost inert and the purpose was only to examine the amount of the plasma concentration of certain chemo-therapeutics which could be freely filtrated. The filtration pressure equalled 35 cm Hg. For the sake of comparison, also sulfanilamide, sulfa-thiazole and sulfapyridine have been ultrafiltrated after application to the same plasma. Series of this kind occur with 3 different kinds of plasmas.

As regards the unstained sulfanilamide derivatives the difference in the values of the cerebrospinal fluid concentrations found in the literature is comparatively extensive. The same applies to the size of the fraction of the sulfanilamide derivatives which can be ultrafiltrated (cp. pages 50—51). This is quite natural, since this fraction varies (at a constant filtration pressure) mainly in proportion to the concentration of the plasma proteins (and particularly the albumin). The concentration of the sulfanilamide derivatives is, within the therapeutic range (2—20 mg per 100 ml), of only subordinate significance with regard to the percentage of bound drug.

Therefore, the published figures hardly permit the conclusion that in a certain case the concentration of the drugs in the cerebrospinal fluid is equal to the freely filtrable plasma fraction or not.

Further, it has not as yet been established that the binding to the plasma proteins determined by means of such model tests precludes a transmission from the blood to the central nervous system. Moreover, this binding may be severed in or next to the membrane separating the blood and the central nervous system.

Therefore, this problem can only be approached by simultaneous ultrafiltration of the plasma and determination of the concentration in the cerebrospinal fluid of a comprehensive material.

Finally, it should be emphasized that these conclusions cannot, in all likelihood, be applied to all the substances occurring in the blood and cerebrospinal fluid. In some of these cases, factors such as the Donnan equilibria, a specific function of the choroid plexus, etc., are assumed to be of decisive importance.

Summary

In order to obtain an explanation of the different concentrations of various sulfanilamide derivatives in the central nervous system, the following items must, *inter alia*, be taken into account:

1. Their binding to the plasma proteins.
2. The existence of a barrier (i. e. signifying differentiated permeability) between the blood, on the one hand, and the central nervous system and the cerebrospinal fluid, on the other.
3. The affinity to the ganglion cells and the rest of the cerebral substance.

The barrier between the blood and the central nervous system seems to exert an obstructive function, with regard to the acid substances. In the ultrafiltrable plasma fraction, at any rate, they are hindered in proportion to the degree of acidity, while basic substances, penetrate. This seems to be established with regard to dyes and, this possibility is, anyhow, not excluded in the case of sulfanilamide derivatives lacking the character of a dye.

Principally the acid dyes are taken up in the ganglion cells, whereas the basic ones are taken up in the rest of the cerebral substance. In all probability, sulfanilamide derivatives lacking the characteristics of a dye are not bound to the cerebral substance or to the ganglion cells.

The weakly acid sulfanilamide is transmitted from the blood to the cerebrospinal fluid and the central nervous system hardly without any obstruction. Sulfapyridine and sulfathiazole, which are more acid, penetrate to a lesser extent, and prontosil soluble does not penetrate at all. Prontosil rubrum which is very slightly acid does permeate the barrier. However, it is bound to the cerebral substance and cannot, therefore, be ascertained in the cerebrospinal fluid.

After injection into the cerebrospinal fluid, prontosil soluble is taken up, similar to other acid vital dyes, in the ganglion cells in particular.

Thus, the degree of acidity and the coupling in a dye play an important part with regard to the passage and localization to the central nervous system of sulfanilamide derivatives.

CHAPTER VII

Own investigations regarding the distribution of prontosil soluble and sulfanilamide in different organs examined by means of chemical methods

Introduction

Considering that some sulfanilamide derivatives have an even distribution in the organism with, on the whole, the same concentration in all the tissues, and others obtain an uneven distribution with high concentrations mainly in the connective tissue (acquired by a linkage to another substance causing a specific affinity to certain tissue structures), and further, since a varying degree of permeability of different sulfanilamide derivatives occurs in the central nervous system, the results are well in agreement with those derived from the chemical extraction of whole organs and tissues or larger pieces of them, as described in Chapter IV.

On account of the lack of information in the literature regarding the contents of prontosil soluble in various tissues, examinations of this kind have been performed. Also the content of sulfanilamide in the tissues after prontosil medication, has been determined in these animals. For the sake of comparison, the sulfanilamide concentration after the application of pure sulfanilamide has been tested in another series of animals. In the latter case, the results conform altogether with those of other authors.

Methods and results

Method. The method, described by RICHTNÉR (1943) and slightly modified by HERBERTS (1944), has been adopted for the determination of sulfanilamide in the tissues.

Since RICHTNÉR has found that the extraction procedure to a great extent causes a conversion of the acetylated derivative to the free form, only the total amount of sulfanilamide has been determined.

The determination of prontosil soluble in the tissues has been performed in the following manner. When the organs have been removed, cut into small pieces and weighed, 1 ml of a 5 per cent solution of NaOH is added. When the tissues have been dissolved after heating for a few minutes in a water bath, 1.6 ml of distilled water is added. After this, it is neutralized with 0.4 ml of 4 N HCl. Then the proteins are precipitated by means of 0.8 ml of a 15 per cent solution of trichloracetic acid. After centrifuging, the extinction of the filtrate is determined in a Pulfrich step-photometer with filter S 50. The concentration in the filtrate was calculated from a standard curve obtained in the same way as the samples. It should be noted that the extinction curves of prontosil soluble differ somewhat with regard to whether the solution is acid or alkaline (STELZER 1939).

0.2 ml whole blood diluted with 3 ml saponin has been employed for the determination of the prontosil concentration in the blood. After precipitation by means of 0.8 ml of a 15 per cent solution of trichloracetic acid, the concentration of the filtrate has been calculated in the same way as in the tissues. By means of admixture tests (with a known amount of prontosil in the blood), the precipitation of proteins has been found not to involve any determinable loss of prontosil.

The sulfanilamide content in the tissues of the animals treated with prontosil has been established in the following way. After having determined the prontosil concentration by means of the photometer, 0.1 ml 4 N HCl has been added to 2 ml of the filtrate. After heating for 30 minutes in a water bath, the sample has been divided into two equal parts. One of them has been diazotized similarly to the procedure used with regard to the sulfanilamide. The other has been diluted in a corresponding way, but without coupling to ethyl- α -naphthylaminehydrochloride. This one has been used as a comparative fluid. When at the conversion of prontosil soluble to sulfanilamide also sodium-2-amino-7-acetamino-1-naphtol-3,6-disulphonate is produced, this compound might, if diazotizable, be assumed to render the values of the sulfanilamide concentrations too high. Since, according to FULLER, this compound is at any rate not diazotizable before hydrolysis, in some animals the sulfanilamide concentrations were determined before as well as after hydrolysis. The values after hydrolysis did not exceed those obtained before hydrolysis with more than 10 per cent. This difference is altogether explained by the conversion of acetyl-sulfanilamide to free sulfanilamide. In vitro experiments, this sodiumsulphonate was not diazotizable, whether before or after hydrolysis by means of the determination methods employed here.

Table 6 a. Concentrations of sulfanilamide after intravenous injection of 0.5 ml. of a 0.75 % solution of sulfanilamide.

Organ	Concentrations in mg per 100 grammes						Mean ratio cone. in tissue conc. in blood	
	1 hour after inj.				2 hrs after inj.			
	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6	1 hour	2 hours
Blood	5.6	6.8	5.6	6.2	5.0	4.4	10	10
Skin	6.4	6.8	4.6	6.4	6.2	5.1	10	11
Lungs	5.3	5.6	5.1	5.1	5.9	6.0	9	13
Muscle	7.7	6.1	6.5	5.6	5.2	4.4	11	10
Kidneys	10.2	13.0	3.8	8.9	4.9	—	15	10
Brain	3.6	5.4	—	—	—	—	7	—

The mean ratios relate to a blood concentration = 10.

The results, which have been collected in Table 6, show that 1 hour after the intravenous injection of 12.5 mg of prontosil, the skin, the lungs and the kidneys have the highest concentration of prontosil among the examined organs. Musculature has a considerably lower concentration, and the brain reveals no traces of prontosil (Table 6 b).

Moreover, it should be noted that also the concentration of sulfanilamide is somewhat higher in the skin and lungs of animals treated with prontosil (higher than in any other organ), than in those treated with the equimolecular dose of pure sulfanilamide. At the same time after the injection of equimolecular doses of sulfanilamide, an almost identical concentration is found in all the examined tissues (Table 6 a).

It should, however, be added that the prontosil concentration found in the skin corresponds to a very much higher concentration in the connective tissue, probably several times as much, since a great part of the weight of the skin is due to the musculature and the epithelium, etc., which do not contain any detectable quantities of prontosil.

Table 6 b. Concentrations of prontosil soluble and sulfanilamide in various tissues after intravenous injection of 0.5 ml. of a 2.5% solution of prontosil soluble.

Organ	Concentrations in mg per 100 grammes												Mean ratio conc. in tissue conc. in blood			
	1 h. after inj.						2 hrs after inj.						1 hour		2 hours	
	Mouse 1		Mouse 2		Mouse 3		Mouse 4		Mouse 5		Mouse 6		P	S	P	S
	P	S	P	S	P	S	P	S	P	S	P	S	P	S	P	S
Blood	8.8	2.8	8.0	1.6	8.0	3.4	8.8	4.0	1.6	2.2	1.0	1.8	10	10	10	10
Skin	38.4	7.4	37.4	9.8	28.0	12.6	26.0	9.6	23.1	8.6	24.0	8.0	39	37	196	42
Lungs	30.0	7.0	21.6	3.6	26.4	6.7	28.2	9.0	16.1	6.8	15.0	7.2	32	23	129	35
Muscle	6.0	0.7	1.6	2.6	4.0	4.2	5.1	3.1	2.4	1.7	1.7	2.1	5	10	17	10
Kidneys	—	—	9.0	7.3	8.6	7.1	22.4	10.0	5.8	7.0	7.2	6.6	16	17	55	34
Brain	0	—	0	2.6	0	—	0	1.5	0	1.7	0	1.7	0	10	0	9

P = Prontosil soluble.
S = Sulfanilamide.

The mean ratios relate to a blood concentration = 10.

Further, it should be pointed out that the values of the concentration in the various tissues are not directly comparable with regard to prontosil, on the one hand, and sulfanilamide, on the other, owing to the considerably faster excretion of prontosil.

However, the conclusion may be drawn from these chemical determinations that prontosil soluble has an uneven distribution in the organism. The pure sulfanilamide on the other hand, is evenly distributed in the body. Finally, a higher concentration of sulfanilamide is obtained in the skin and lung by treatment with prontosil than by the administration of the equimolecular dose of pure sulfanilamide.

Summary

The uneven distribution of prontosil in the tissues, observed in the fluorescence microscope, is confirmed by the determination of the prontosil concentration in the various organs and tissues by means of chemical methods. As was to be expected, the skin and the lung had the highest prontosil concentration, followed next by the kidney, the lowest concentration occurring in the musculature. No traces of prontosil soluble are ascertainable in the brain.

As regards sulfanilamide, the concentration in the skin, the lung and the musculature was approximately identical, whereas it was higher in the kidneys and somewhat lower in the brain.

After the administration of prontosil also higher concentrations of sulfanilamide were obtained in the skin and lung than after sulfanilamide medication (equimolecular doses), while the concentration in the blood and musculature was somewhat lower.

CHAPTER VIII

Own investigations regarding the possibilities of directing drugs to certain tissues

Investigations regarding the prerequisites for directing sulfanilamide derivatives to elastin and collagen

The affinity to elastin and collagen of prontosil soluble having been proved, the question of the particular factors necessary for producing this affinity arises.

The factors which may be expected to play a part in this connection are as follows: 1) the drug's character of an azo-dye, 2) the acid or basic nature of the substance at the pH of the blood, and 3) the acid or basic groups of the compound coupled to the sulfanilamide derivatives by means of an azo linkage.

The question whether the acid or basic nature of the substance is of any significance in this connection was first subjected to analysis. Accordingly, chrysoidine, which is a purely basic substance and very closely related to prontosil rubrum, was injected intravenously (0.5 ml. of a 2.5 per cent solution). Chrysoidine was found not to have any affinity to elastin and collagen.

Since prontosil rubrum is stated to be a basic dye, it was necessary to examine this substance too. As it is not easily dissolved and therefore unsuitable for injection, it was baked into bread and given to the animals during periods of 2—4 hours. When the animals were sacrificed, the macroscopical examination revealed the ears of the mice to be yellow. Microscopically, prontosil rubrum was found to have the same affinity to elastin and collagen as prontosil soluble. In order to examine

whether the prontosil rubrum and prontosil soluble were positively or negatively charged, electrophoresis experiments were performed at the pH of the blood. Such tests have been carried out earlier by BENNHOLD (1938), who discovered that prontosil follows the albumin, and by SCHÖNHOLZER (1940), who arrived at identical results. However, since the former did not state whether he had studied prontosil rubrum or soluble, or both, and the latter merely writes that he examined »prontosil rubrum soluble», it remained unclear whether both the preparations had been tested and, if so, whether they both behaved similarly.

These electrophoresis examinations have been performed by B. OLHAGEN. He employed an apparatus constructed by TISELIUS-SVENSSON. The electrophoresis is made at a temperature of $+0.5^{\circ}\text{C}$ in a serum dilution of 1:4, in a phosphate buffer, at an ion strength of 0.1, the pH being 7.68. The potential gradient (F) equalled 7 volt/cm. The drug concentration was 0.02 per cent.

Prontosil rubrum, as well as prontosil soluble, were found to be negatively charged at the pH of the blood, moving towards the anode, one part more rapidly than the protein gradients, and another part at an equal pace. Thus, both drugs act as acid dyes at the pH of the blood.

The acid, i. e. electro-negative, nature of an azo-dye at the pH of the blood seems to be a *sine qua non* for the staining of elastin and collagen. The fact that sulfathiazole, which is fairly strongly acid, does not have this affinity, serves to prove that the azo linkage is of significance and that merely the acid nature of the substances will not suffice. On the other hand, sulfanilamide derivatives may, of course, possibly be combined in some other way than in the azo linkage, thereby obtaining an affinity to certain tissue structures. However, this question will not be entered upon here.

After having established the affinity of at least some acid azo-dyes to elastin and collagen, it remained to be determined whether any importance had to be attached to the particular group producing the acidity.

Thus, prontosil rubrum and prontosil soluble are acid, the former owing to the sulfonamide group, and the latter owing to the sulfonic acid groups + the sulfonamide group. Since preparations with carboxylic groups already existed in salazothiazole and salazopyrin, they were subjected to a test. They, too, were found to have an affinity to elastin and collagen. However, it should be noted that these preparations also have the acid sulfonamide group in sulfathiazole and sulfapyridine (cp. folded page at back with formulae). This group has alone sufficed to produce an affinity in prontosil rubrum. Accordingly, this merely serves to demonstrate that the affinity does not change when the acid groups of the compound coupled to the sulfanilamide derivatives by means of an azo linkage are changed.

Also a few common azo-dyes, viz. Congo red and trypan-blue, have been tested and found to have the same affinity. As regards the dyes neutral red and phenol red, the former being stated to be a basic dye and the latter an acid one, neutral red was found to lack an affinity to elastin and collagen, while phenol red had this affinity. Obviously, even acid dyes other than azo-dyes may have an elastin and collagen affinity. However, the determining factors in these cases have not been closely studied. The fact that, at any rate, all the tested acid azo-dyes had an elastin and collagen affinity has been allowed to suffice in this connection.

The elastin affinity of several acid dyes and, above all, of the azo-dyes has been demonstrated earlier by, among others, KIYONO & AMANO (1937/38) Still, they merely mentioned one basic dye which was assumed to have an elastin affinity, viz. Bismarck brown. Other investigators (SEKI, VON MÖLLENDORFF), describing vital staining with this dye, have not specified whether or not an elastin affinity existed.

SEKI (1933) reported that this dye can be recharged and, since it is rarely pure but consists of a mixture of different compounds, it is not improbable that the exceptional character of this dye is merely imaginary. In my own investigations, Bismarck brown has not had an affinity to elastin and collagen.

ROMAN (1918) described a vital staining of elastin and collagen obtained by means of thienyl-chinolin-carbonic acid, i. e. a compound in close relation to atofan, in which the phenyl group has been substituted with a tiofen group.

The question whether the affinity to elastin and collagen should be interpreted as an adsorption or a salt bond is nowadays of minor interest, since the conception is steadily gaining ground that, in principle, no difference occurs between these two forms of staining. Thus, KARRER (1942) writes in the chapter regarding the azo-dyes, as follows:

»The difference between the adsorption and salt formation theory has anyhow lost in conspicuity and significance ever since the adsorption was interpreted as a process, where the valence powers derived from the atom lattice of the adsorbent have been conceived to cause the adsorption, since these valence powers are identical with normal chemical valences.

Thus, the fixation of dyes in wool and silk is, no doubt, due to such free lattice valences. Moreover, through the electrostatic nature of these valences (positive and negative), also the affinity to basic and acid dyes becomes explicable.»

Particularly important in this connection is, apparently, above all that the binding to elastin and collagen is sufficiently loose to permit the liberation of prontosil. This is evident from the fact that the high concentrations, which occur in the elastic and collagenous structures, as late as an hour after the intravenous injection, have altogether disappeared after another 3 hours. Further, prontosil can easily be washed out of elastin and collagen. One drop of distilled water on a section will suffice to cause the prontosil to diffuse within a few minutes.

The pronounced looseness also of the binding to the plasma proteins is noted, inter alia, by the liberation of prontosil by shaking with charcoal. The adsorption capacity of the charcoal is thus found to be stronger than that of the plasma proteins. This has been established earlier with regard to other sulfanilamide derivatives (KIMMIG and WESELMANN 1941) and some azo-dyes [EHRSTRÖM (1936), GOTTLIEB and LUDWIG (1937)].

In order to examine the accuracy of this principle, viz. that the acid azo dyes have an elastin and collagen affinity, it was considered appropriate to couple the sulfathiazole in an azo linkage in the same way as sulfanilamide is coupled in prontosil rubrum and soluble. Since acetamino-naftol-disulfonic acid was unobtainable, amino-naftol-disulfonic acid was used instead.

The preparations, which have been prepared by AB Astra, have been called Prep I and II.¹

When these compounds were injected intravenously, they were found to have, as expected, an elastin and collagen affinity. In electrophoresis experiments, also Prep. I moved towards the positive electrode. In addition, another observation of interest was recorded. Prep. I was excreted more slowly than Prep. II and remained after 4 hours in high concentrations in the connective tissue. This is, in all probability, due to factors such as solubility, the size of the particles, diffusion speed, etc., as experiences from the comprehensive literature regarding vital dyes will indicate. In this particular case, these conditions involved a considerably slower excretion through the liver of Prep. I than of prontosil soluble, prontosil rubrum or Prep. II, causing high concentrations also in the liver cells. On the other hand, Prep. II, as well as prontosil soluble, pass rapidly through the cells of the liver and in low concentrations, attaining high concentrations only in the biliary capillaries.

**A description of sections from animals treated with 0.5 ml 2.5 %
sol. of Prep. I intravenously.**

(All the animals were killed at the same time of the day.)

2 minutes after the injection (3 animals).

The hepatic cells were of a marked brown colour in the region next to the central vein. The staining weakens successively towards the periphery. The nuclei were unstained. Regions occur between these brown parts where the hepatic cells have a blue fluorescence with a faint yellowish gleam.

¹ Astra is, at present, producing the sodium salt of sulfathiazole-azo-acetamino-naftol-disulfonic acid as well as a series of other sulfathiazole and sulfapyridine azo derivatives.

15—60 minutes after the injection (6 animals).

Liver. Still an even yellow-brown colour. Nuclei unstained. The granules of the v. Kupffer cells still have a yellow colour.

The cells in the *intestine* are also to a large extent a yellow-brown.

4 hours after the injection (3 animals).

The hepatic cells still fluoresce in a marked yellow-brown colour. However, the brown colour is now less pronounced round the central vein. Thus, the picture is reversed as compared to the one obtained 2 minutes after injection.

Otherwise, the localization with regard to elastin and collagen coincides with that of prontosil soluble. Prep. I, however, is retained in almost equally high concentrations 4 hours after the injection as 15 minutes after.

Thus, the excretion rapidity can be varied by changing the compound which is coupled to the drugs in an azo linkage. Further, not only can the depot effect in the connective tissue be prolonged, but there may also be a theoretical possibility of higher concentrations even in the epithelial cells, where the drugs are resorbed and excreted, or, in fact, in all the epithelial cells into which the drugs may pass.

**Do any other theoretical possibilities exist of directing
drugs to certain tissues?**

As pointed out before, there should be several possibilities of coupling, apart from the azo combinations. The literature dealing with the subject of vital staining might be expected to indicate some fundamental lines to pursue in the search for suitable combinations. However, the results derived from this abundant literature do not appear immediately appropriate for this particular purpose. Naturally, a discussion of the details cannot be entered upon in this paper. A few experiences will, nevertheless, be submitted. So far, the capacity of vital staining has been found to be an exceedingly complicated matter in some substances, being more dependent on the physical properties of the compound than on its chemical properties (HERTWIG, KIYONO, VON MÖLLENDORFF, SEKI, GICKLHORN, KELLER).

The factors which have been found to be of particular interest are, as follows:

1. The degree of dispersion. The smaller the size of the molecules and the particles, the easier the penetration of the substances into the cells.
2. The electrical charge.
3. The solubility. This plays a part, *inter alia*, with regard to the time during which the substances remain in the cells. The less the solubility, the slower the excretion.
4. Lipoid-solubility is not a necessity for the staining of cells rich in lipoids.

However, the interplay of these factors is such a complicated matter that, even if all are known, it is often difficult to predict, with any higher degree of certainty, the localization in the organism. Moreover, attention has generally mostly been directed to the vital staining of different cells. Accordingly, data are, as a rule, lacking regarding the possible vital staining of substances such as elastin and collagen.

The mere fact that various cells and cellular structures have been successfully stained with different vital dyes, however, indicates great possibilities of development in this method of directing pharmacologically active substances straight to the particular place where a higher concentration is wanted. Finally, it should be emphasized that the combination of freezing-drying fixation and fluorescence microscopy would widen the basis of future research with regard to vital staining, considering the fact that the same conditions apply to this combination as to histo-pharmacological studies, as previously stated, and, also, that many of the vital dyes in common use show an extremely marked fluorescence.

Summary

An elastin and collagen affinity has been proved to be a property inherent in all acid azo-dyes tested up til now, viz. prontosil solubile, prontosil rubrum, salazothiazole, salazopyrin, Prep.

I and II, Congo red and trypan blue. Accordingly, by coupling substances in such a linkage they can be directed to the connective tissue. By varying the compound to which the sulfanilamide derivatives are coupled by means of the azo linkage the excretion rapidity can be affected. In this way, high concentrations can also be obtained in, for instance, the liver. Finally, through the further application of these principles, various future potentialities with regard to the direction of drugs other than chemo-therapeutics have been indicated.

CHAPTER IX

General discussion

Prontosil rubrum and soluble, as distinguished from pure sulfanilamide, have been found to show an extremely irregular distribution in the tissues with high concentrations particularly in the connective tissue. This is due to the pronounced affinity of these compounds to elastin and collagen. The question arises whether this explains the better effect obtained by the use of prontosil drugs in the case of some infections, such as erysipelas, than by a dose of sulfanilamide corresponding to the amount of sulfanilamide reductively converted by prontosil. The answer is, in my opinion, in the affirmative.

It should be evident, from the examinations performed, that higher concentrations of sulfanilamide occur in, for instance, the skin by the injection of sulfanilamide coupled in an azo linkage, than by a supply of equimolecular doses of sulfanilamide in a free form. This notwithstanding that the particular prontosil preparation employed (prontosil soluble) is unsuitable for a comparison of this kind because of its speedy excretion. At the time of the test (1 hour after the injection) a large amount had already been excreted unchanged, while the pure sulfanilamide is not excreted so quickly.

The concentrations of prontosil occurring in the connective tissue are, in all likelihood, calculated to exceed those obtained by the supply of pure sulfanilamide to such an extent as to be several times higher. For it is obvious, that from the ascertained prontosil quantity, the main part is localized to the elastic and collagenous fibers. Since a large part of the skin consists of musculature, fat, glands, epithelium, nerves, etc., the prontosil concentration in the elastin and collagen is, very likely, several

times higher than that of the entire analyzed piece of skin (cp. Fig. 5).

The dose of sulfanilamide, administered in a pure form, is probably more diffusely distributed all over the skin. The distribution in the skin of the sulfanilamide produced by reduction of prontosil is unknown. However, the highest concentration of sulfanilamide probably occurs in the surrounding parts close to the high prontosil concentration. *Thus, the sulfanilamide concentration after the prontosil treatment becomes several times higher than in treatment with equimolecular sulfanilamide doses.* In addition, a depot effect of prontosil is obtained owing to the elastin and collagen affinity. This is due to the fact that prontosil has been demonstrated to remain for a longer time in these tissues than elsewhere.

This suffices, it seems to me, to explain the better effect gained by the prontosil drugs in infections localized to the connective tissue, in particular, viz. erysipelas, than by pure sulfanilamide, even though prontosil may be assumed to exert its action only by the sulfanilamide obtained by reductive conversion.

In addition, the forementioned facts throw light upon the relative absence of toxic effects from prontosil preparations. As was to be expected, from the fluorescence microscopical picture, and from the quantitative examinations of tissues and blood, the prontosil concentration in the blood and in the organs deficient in elastin and collagen, is considerably lower than that obtained with equimolecular doses of sulfanilamide, this notwithstanding the higher skin concentration in the former case. Apparently, the toxic effects are due to the concentrations in organs and tissues other than the skin, viz. blood, brain, etc. *These toxic effects have, therefore, to some extent, been avoided by means of the azo linkage.*

It follows, as a matter of course, that in the case of prontosil an examination of the blood concentration, for the purpose of facilitating an estimation of the therapeutical effect, is not worth while. Still, it may be justified in connection with sulfanilamide derivatives with an even and diffuse distribution. *Since the concentration at the site of the infection determines the chemo-*

therapeutic effect, the prontosil preparations must, in the event of infections in the connective tissue, be regarded as ideal from the point of view of localization. This localization is all the more valuable on account of the particularly frequent occurrence of infections in this tissue.

An additional conclusion may be drawn from the results obtained with prontosil soluble. Sulfanilamide passes quite easily into the central nervous system where it attains concentrations approximately equal to those of the blood. On the other hand, prontosil does not penetrate into the central nervous system. (Further, the sulfanilamide concentration in the cerebrospinal fluid is very low at a prontosil therapy.) *Thus, substances not wanted in the central nervous system may be kept away by coupling in an acid azo compound.*

However, the prontosil preparations are not so satisfactory from a therapeutical point of view, since sulfanilamide is not the most active substance among the known sulfanilamide derivatives. *When it was found that, in all probability, the majority of the acid azo-dyes had an elastin and collagen affinity, an attempt to couple sulfathiazole in an acid azo dye suggested itself, with a view to exploiting the good chemo-therapeutical effect obtained by sulfathiazole, as well as the favourable localization properties of the azo linkage.* For this reason, inter alia, Prep. I and II were produced. Unfortunately, a sufficient quantity of raw materials has, as yet, not been available for testing the chemo-therapeutical effect of the drugs on localized infections. So far, all that can be said regarding these preparations is that they have the desired localization.

These two compounds, however, offer another point of interest in this connection. *By varying the compounds which are coupled to the sulfanilamide derivatives by means of the azo linkage, the excretory rapidity of the preparation can be changed.*

Thus, Prep. I disclosed considerably slower excretion than Prep. II. In this way, high concentrations occurred, inter alia, in the hepatic cells. This offered at least a possibility of pro-

ducing a more favourable chemo-therapeutical effect also in these cells.

The azo linkage is also of value when salazopyrine and salazothiazole preparations are concerned. These drugs were suggested by N. SVARTZ chiefly for application in polyarthritis. *With these drugs comparatively high concentrations, attained by means of the azo linkages, of salicylic acid as well as sulfathiazole and sulfapyridine undoubtedly occur in the elastic cartilage and in the connective tissue surrounding the joints (joint capsules, tendons, etc.).*

Nevertheless, the coupling to an acid azo-dye is not only useful when sulfanilamide derivatives are to be directed to the connective tissue in order to attain high concentrations and an accumulative effect. It should also be applicable for the same purpose with other drugs. It is only necessary that the drug is diazotizable. The azo compound, in particular, is very useful since it is converted in the organism, during which process the active substance is liberated. It is, of course, possible that some of these substances are also active as azo compounds.

Finally, yet another way of directing drugs to a definite organic system may be added. Substances with sufficiently large particles for storing in the R. E. S., should be obtainable by coupling the effective substance in the azo linkage and selecting a suitable compound for this purpose in order to obtain high concentrations in this system.

Future research will probably discover means of directing drugs also to other tissues, tissue structures, cells and cellular parts by coupling in other compounds than the azo ones.

Survey of the results

Part I

The Altmann freezing-drying method is generally applicable in fixation for histo-pharmacological purposes.

Fluorescence microscopy is, in a great number of instances, a useful method for detecting drugs in the tissues particularly in connection with a new heating procedure, described by the author, which produces a contrast between the fluorescence of drugs and that of tissues in several cases where a contrast has otherwise not occurred.

Part II

The fact that prontosil rubrum and prontosil soluble appear to have a better effect in certain localized infections, such as erysipelas, than corresponding to the reductively converted sulfanilamide is, in all likelihood, due to the varying behaviour of these drugs when introduced into the organism. The sulfanilamide spreads, on the whole, evenly and diffusely over the entire organism, while the prontosil preparations have an extremely irregular distribution with high concentrations chiefly in the pulmonary tissue, the connective tissue, the elastic cartilage, the tendons and ligaments, and low concentrations in the remaining organs and tissues. No storing has been ascertainable in the reticulo-endothelial system (R. E. S.). Since the concentration at the site of the infection is important with regard to the chemotherapeutical effect, and considering also that, inter alia, the skin contains not only prontosil at high concentrations, but also sulfanilamide in higher concentrations than those obtained by injection of equimolecular doses of pure sulfanilamide, prontosil compounds appear capable of producing a superior effect particularly in infections such as erysipelas.

Intravenous injections cause higher concentrations in the skin than local application in the form of an ointment, at any rate when the surface of the skin is intact.

The effect of prontosil in the case of pneumonia, less satisfactory than might be expected considering the high concentrations in the lungs, may depend on the insignificant effect of sulfanilamide on pneumococci.

The comparatively low concentration of sulfanilamide and prontosil in the blood and other tissues at prontosil treatment may explain the comparative rarity of the toxic effects at prontosil therapy.

The explanation of the uneven distribution of the prontosil compounds lies in their strong affinity to elastin and collagen.

It has been clearly shown that this affinity is dependant on the acid azo-dye character of these drugs, this affinity appertaining to all the acid azo compounds so far tested, but not to the basic ones.

Thus, thanks to the azo linkage, the salazopyrine and salazothiazole preparations, for instance, may at the reduction of the azo linkage in the tissues, attain high concentrations of salicylic acid, as well as sulfathiazole and sulfapyridine in, e. g., the joint capsules, tendons and ligaments.

This method of directing sulfanilamide derivatives, by coupling in acid azo-dyes, straight to the tissues rich in elastin and collagen was applied to sulfathiazole (which in its pure form has a diffuse distribution in the organism) for the purpose of obtaining an easily soluble preparation, which combined the good chemo-therapeutical properties of sulfathiazole with the favourable localization in the tissues of the azo-dyes.

The compounds thus produced possessed the desired properties with regard to localization.

The chemo-therapeutical effect has not been provable owing to the lack of raw materials.

By varying the compound which is coupled to the drug by means of the azo linkage, the diffusion capacity, the solubility, the rapidity of excretion, etc., of the substance may be changed.

In this way, high concentrations may also be attained in other organs, such as the liver, the reticulo-endothelial system, etc.

The fact that acid azo-dyes do not penetrate into the central nervous system, renders possible the detoxication of substances which have a toxic effect on the central nervous system.

One of the main factors of significance with regard to the passage to the central nervous system is, apparently, the binding to the plasma proteins. In addition, the occurrence of a barrier is probable between the blood and the central nervous system, signifying a differentiated permeability of different substances in relation to their degree of acidity.

The possibility of employing the linkage in azo compounds and other substances in order to direct drugs to different organs and tissues has been discussed.

References

ALEXANDER F. Quart. J. Exper. Physiol. 1943: 32: 21.

ALTMANN R. Die Elementarorganismen und ihre Beziehungen zu den Zellen. 2 Aufl. Leipzig, 1894.

ANDERSEN A. H., MÖLLER K. O., SIMESEN M. H. Arch. f. Exper. Path. u. Pharmakol. 1942: 199: 528.

-- SIMESEN M. H. Acta med. Scandinav. 1943: 114: 104.

ANDERSON T. Lancet 1939: 237: 257.

— Brit. M. J. 1939: 1: 716.

BECKER W. Dermat. Wchnschr. 1937: 104: 221.

BECKER-CHRISTENSEN P., SIMESEN M. H. Nord. med. 1941: 12: 3147.

BELL P. H., ROBLIN R. O. J. Am. Chem. Soc. 1942: 64: II: 2905.

BENNHOLD H., KYLIN E., RUSZNYÁK St. Die Eiweisskörper des Blutplasmas, Dresden 1938.

BEQUIRISTAIN B. Chem. Zentralbl. 1942: 1: 2800.

BOCK E., OESTERLEN M. Zentralbl. f. Bakt. 1939: 143: 306.

BORELL U., TROELL L. Acta med. Scandinav. 1943: 115: 587.

BOSSE, G., Deutsch. med. Wchnschr. 1938: 64: 116.

BRATTON A. C., MARSHALL JR. E. K., J. Biol. Chem. 1939: 128: 537.

BROMAN T. Arch. f. Psychiat. 1941: 112: 290 and 309.

BUCHHEIM, DRECHSLER, Zentralbl. f. Chir. 1938: 2063.

BUTTLE G. A. H., GRAY W. H., STEPHENSON D. Lancet 1936: I: 1286.

CARNOT P., COQUOIN R. Compt. rend. Soc. de biol. 1926: 94: 850.

CHRISTENSEN B. CHR., Nord. med. 1939: 2: 1379.

CLARK, A. J., The mode of action of drugs on cells. London 1933.

— Handbuch der experimentellen Pharmakologie. Begr. von A. Heffter, Erg.-Werk. 4. 1937.

COLEBROOK, L., BUTTLE, G. A. H., O'MEARA, R. A. Q. Lancet 1936: II: 1323.

— KENNY, M. Lancet 1936: I: 1279.

DAVIS, B. Science 1942: 95: 78.

DHÉRÉ, CH. La fluorescence en Biochimie, Paris 1937.

DOMAGK, G., HEGLER, C. Chemotherapie bakterieller Infektionen. Leipzig 1944.

DONIACH, I., MOTTRAM, J. C., WEIGERT, F., Brit. J. Exper. Path. 1943: 24: 1.

DUTT, S. Journ. of the Indian Chem. Soc. 1930: VII: 505.

EHRSTRÖM, M. Ch. 1936. Acta med. Scandinav 1936: 88: 517.

ELLINGER, P. Cambridge Philos. Soc. Biol. Rev. 1940: 15: 323.

— Biochem. J. 1938: 32: 376.

— KOSCHARA, W. Ber. deutsch. chem. Ges. 1933: 66: 315.
Ibid. 1933: 66: 808 and 1411.
Nature 1934: 133: 553.

ENGEL, C. Acta brev. Neerland. 1938: 8: 120.

v. EULER, H., HELLSTRÖM, H., ADLER, E. Ztschr. f. vergleich. Physiol. 1935: 21: 739.

FALKENSTEIN, F. 1932. Dermat. Wehnschr. 1932: 50: 1785.

FEINSTONE W. H., BLISS E. A., OTT E., LONG P. H. Bull. Johns Hopkins Hosp. 1938: 62: 565.

FISCHL, SCHWENK E. Klin. Wehnschr. 1932: 11: 1114.

— SINGER E. Ztschr. f. Hyg. u. Infektionskr. 1935: 116: 348.

FOLEY J. A., YASUNA E. R. J. A. M. A. 1940: 115: 1330.

FRANKLIN A. J., ALLEN J., Franklin Inst. 1940: 230: 263.

FRIEDEMANN U., J. Immunol. 1937: 32: 97.

FRISK A. Acta med. Scandinav. 1943: Suppl. CXLI.

FULLER A. T. Lancet 1937: I: 194.

GERSH I. Am. J. Physiol. 1934: 108: 355.

— CASPERSSON T. Anat. Rec. 1940: 78: 303.

GICKLHORN J., Ergeb. d. Plupiol. 1931: 31: 388.

— KELLER R., Methoden der Bioelektrostatistik. Handbuch der biologischen Arbeitsmethoden. Hrsg. von E. Abderhalden. V: 2: 1.

GRAY P., GIRARD A. Presse méd. 1936: 44: 1775.

GMELIN L. München. med Wehnschr. 1935: 82: 221.

GOLDMANN E. E. Abh. d. Kgl. Preuss. Akad. d. Wiss. Math.-naturw. Kl. 1913: 1.

GOTTLIEB H., LUDWIG H. Ztschr. f. klin. Med. 1937: 131: 358.

GRAFFI A. Ztschr. f. Krebsforsch. 1939: 49: 477.

— Ibid. 1940: 50: 196. Ibid. 1941: 52: 165 and 235.

GÜNTHER H. Ztschr. f. Krebsforsch. 1941-2: 52: 57.

HAAS H. Arch. f. exper. Path. u. Pharmakol. 1941: 197: 57.

HACKMAN CHR. Medizin and Chemie. Herausgeg. von Igefa 1942: 4: 134.

HAITINGER M. Die Fluoreszenzanalyse in der Mikrochemie. Wien und Leipzig 1937.

— Fluoreszenzmikroskopie. Ihre Anwendung in der Histologie und Chemie. Leipzig 1938.

— FEIGL F., SIMON A. Mikrochemie 1932: 10: 117.

Handbooks of Chemistry and Physics 1944.

Handwörterbuch der Naturwissenschaften. 1934.

HARTL K., Deutsch. med. Wehnschr. 1936: 62: 1641.

HELANDER S., Upsala lärkaref. förh. 1943: 49: 245.

HELANDER S., *Acta physiol. Scandinav.* 1944: 8: 134.
— *Nature* 1945: 155: 109.
— RICHTNÉR N., SJÖSTRAND F., SJÖSTRAND T., 1945. Unpublished paper.
HERBERTS S. *Acta oto-laryng.* 1944 Suppl. LV.
HERTWIG, G.: *Handbuch d. mikroskop. Anat. d. Menschen.* Hrsg. von W. v. Möllendorff. Bd 1: 1: 47. 1929.
HIRT A., WIMMER K. *Klin. Wehnschr.* 1939: 18: 733.
— *Klin. Wehnschr.* 1939: 18: 765.
HOFFHEINZ S., *Deutsche med. Wehnschr.* 1940: 66: 626.
HOFSTÄTTER R., SCHWARTZ J., *Monatschr. f. Geburtsh. u. Gynäk.* 1938: 107: 196.
HOYNE A. L., WOLF A. A., PRIM L. J. A. M. A. 1939: 113: 2279.
HUBBARD R. S., BUTSCH W. L. *Proc. Soc. Exper. Biol. & Med.* 1941: 46: 484.
ISHIKAWA Y. *Klin. Wehnschr.* 1939: 18: 1251.
v. JANCZO N. *Klin. Wehnschr.* 1932: 11: 689.
JOSEPH A. *Compt. rend. Assoc. d. Anat.* 1927: 22: 119.
KARRER H. *Lehrbuch der organischen Chemie*, Leipzig 1942.
KEY A., RETZIUS G. *Studien in der Anatomie des Nervensystems und des Bindegewebes.* Stockholm 1875.
KIMMIG J., WESELMANN H. *Arch. f. Dermat. u. Syph.* 1941: 182: 436.
KIYONO K., AMANO S. *Acta Schol. Med. Univ. Imp. Kioto*, 1937/38: 20: 17.
— SUGIYAMA S., AMANO S. *Ibid.* 1938: 21: 1.
KLEE PH., RÖMER H., *Deutsche med. Wehnschr.* 1935: 61: 253.
KÖNIG W., *Deutsch. med. Wehnschr.* 1939: 65: 601.
LAVIETES P. *Biol. Chem.* 1937: 120: 267.
LEVADITI C. *Presse méd.* 1938: 30: 672.
— VAISMAN A., *Compt rend. Acad. d. sc.* 1935: 200: 1694.
 Compt rend. Soc. de biol. 1935: 119: 946.
 Presse méd. 1935: 43: 2097.
— BEQUIGNON R., REINIÉ L. *Compt. rend. Soc. de biol.* 1938: 128: 355.
LEY H. *Angew. Chem.* 1908: 21: 2027.
— Fluorescenz und chemische Konstitution. *Handbuch d. Physik* 1929.
 Bd. 21.
— Fluorescenz; *Handwörterbuch der Naturwissenschaften* Bd 4: 1934.
LITCHFIELD JR. J. T., WHITE H. J., MARSHALL JR. E. K. *J. Pharmacol. & Exper. Therap.* 1941: 72: 291.
LÖHE H., *Mcd. Klin.* 1940: 36: 150.
LONG P. H., BLISS E. A. *J. A. M. A.* 1937: 108: 32.
— *The Clinical and Experimental Use of Sulfanilamide, Sulfapyridine and Allied Compounds.* New York 1939.

MC KEE G., HERMAN F., BAER R. L., SULZBERGER M. Science 1943:
 28: 1642.
 — J. Lab. Clin. Med. 1943: 98: 66.
 MAHER F. T., CAMP W. J. R. J. Lab. & Clin. Med. 1938—39: 24: 1198.
 MANN G. *Physiological Histology*. Oxford 1902.
 MARSHALL E. K. JR., EMERSON K., CUTTING W. C. J., J. Biol. Chem.
 1937: 122: 263.
 — J. Pharm. & Exper. Therap. 1937: 61: 191 and 196.
 — CUTTING W., COVER, Bull Johns Hopkins Hosp. 1938: 63: 318.
 MEYER ZU HÖRSTE G. Klin. Wehnschr. 1936: 15: 1602.
 MIESCHER G., Dermatologica 1941: 83: 50.
 MÖLLENDORFF W. v. Methoden zum Studium über vitale Färbungen an
 Tierzellen. Handbuch der biologischen Arbeitsmethoden Hrsg. von
 E. Abderhalden V: 2: 1: 97.
 NELSON J., RINZLER H., KELSEY M. P., J. A. M. A. 1939: 112: 1044.
 NITTI F., BOVET P. Compt. rend. Soc. de. biol. 1935: 119: 1277.
 OBERDALHOFF M. Arch. f. Gewerbepath. u. Gewerbehyg. 1939: 9: 435.
 PATZELT K., SHAIRER E. Klin. Wehnschr. 1940: 19: 1251.
 POLONY B. Dermat. Wehnschr. 1939: 108: 188.
 POPPER H. Proc. Soc. Exper. Biol. & Med. a) 1940: 43: 133.
 — Proc. Soc. Exper. Biol. & Med. b) 1940: 43: 234.
 PRINGSHEIM P. *Fluorescenz u. Luminescenz*. Berlin 1928.
 — Luminescenz. Handbuch d. Physik 1929. Bd. 21.
 v. QUERNER F. R. Naturwissensch. 1932: 20: 908.
 — Klin. Wehnschr. 1935: 14: 1213.
 — STURM K. Anat. Anz. 1934: 78: 289.
 RADLEY J. A., GRANT J. *Fluorescence analysis in ultra-violet light*. London. 1935.
 REINHOLD J. G., FLIPPIN H. F., DOMM A. H., POLLACK L., Am. J. M.
 Sc. 1944: 207: 413.
 RICHTNÉR N. S. Acta oto-laryng. Suppl. XLIX 1943.
 ROMAN B. Corr.-Bl. f. Schweizer Ärzte 1918: 48: 1638.
 SEKI M. Folia anat. Japonica 1932: 10: 621.
 — » » 1933: 11: 1.
 — » » 1934: 12: 11.
 — Zschr. f. Zellforsch. u. mikr. Anat. 1933: 18: 1.
 — » » » » 1933: 19: 238.
 SCHREUS H. T. Deutsche med. Wehnschr. 1940: 19: II: 790.
 SCHÖNHOLZER G. Klin. Wehnschr. 1940: 19: II: 790.
 SHAIRER E., PATZELT K., RECHENBERGER J., GOCKEL H. Virchows
 Arch. f. path. Anat. 1939: 305: 360.
 — Virchows Arch. f. path. Anat. 1940—41: 307: 124.
 SIMESEN M. H. Nord. Med. 1939: 22: 1663.
 — Arch. f. exper. Path. u. Pharmacol. 1940: 197: 12.

SITTENAUER L. Zentralbl. f. Chir. 1940: 67: 503.
SJÖSTRAND F. Über die Eigenfluorescenz tierischer Gewebe mit besonderer Berücksichtigung der Säugetierniere. Stockholm 1944.
— SJÖSTRAND T. Skandinav. Archiv f. Physiol. 1938: 79: 156.
SNODGRASS W. R., ANDERSSON T. Brit. med. Journ. 1937: II: 101.
— Ibid., 1937: II: 1156.
— RENNIE J. L. Brit. Med. Journ. 1938: II: 399.
SOFUE N. Jap. J. M. Sc. IV Pharmacol. 1940: 12: 1.
— Ibid. 1940: 13: 1.
SPITZER B., LOOS ST. Jahrb. f. Psychiat. u. Neurol. 1934: 50: 309.
STELZER S., Arch. f. exper. Path. u. Pharmacol. 1940: 194: 133.
STRÖBEL Wien. Klin. Wehnschr. 1939: 954.
STRAUSS E., LOWELL F. C., TAYLOR F. H. L. Finland M., Ann. Int. Med. 1941: 14: 1360.
SVARTZ N. Nord. Med. 1941: 9: 554.
— Acta med. Scandinav. 1942: 110: 577.
TAYLOR A., ÅGREN G., Acta physiol. Scandinav. 1940: 1: 79.
TONNDORFF Med. klin. 1936: II: 1307.
TOOMEY J. A., Ann. Int. Med. 1938: 12: 166.
TRÉFOUËL J., TRÉFOUËL MME., J., NITTI F., BOVET D., Compt. rend. Soc. de biol. 1935: 120: 756.
TURCHINI J., Compt. rend. Assoc. d. Anat. 1926: 21: 548.
UNSHELM E., Med. Welt 1937: 11: 660 and 721.
VOLAVSEK W. Wien. klin. Wehnschr. 1940: 53: 996.
VONKENNEL J., SCHMIDT W., Klin. Wehnschr. 1939: 18: 150.
WHITBY L. E. H. Lancet 1937: I: 1517.
WIKANDER T. Farmaceut. Revy 1931: 30: 393—438.



ACTA PHYSIOLOGICA SCANDINAVICA
VOL. 10. SUPPLEMENTUM XXX.

From Wenner-Gren's Institute for Experimental Biology
University of Stockholm

THE METABOLIC EFFECT AND THE
BINDING OF CARBON DIOXIDE
IN BAKER'S YEAST

By

KNUT M. BRANDT

Stockholm

1945

Corrections

to Acta Physiologica Scandinavica,

Vol. 10. Supplementum XXX:

The Metabolic Effect and the Binding of Carbon Dioxide in Baker's Yeast.

Page 18, lines 8 and 12, read HERAEUS for HEREAUS.

» 24, line 1, read »pyruvic» for »fumaric».

» 93, » 8, » »H₃PO₄» for »H₂PO₄».

» 98, » 8, » »perchlorate» for »persulphate».

» 125, » 36, » »and» for »although».

» 153, » 1, » »for» for »or».

» 158—162, line 1 in all figure texts, read 3.00 for 0.30.

» 195, line 10, read FISHER for FISCHER.

CONTENTS.

	Page
Acknowledgements	5
General Introduction	7
<i>Chapter I. The Effect of Carbon Dioxide on Living Cells and its Significance in Biological Processes. A Review</i>	<i>12</i>
A. The importance of carbon dioxide for the growth of micro-organisms	12
B. Carbon dioxide fixation and reduction in autotrophic and heterotrophic cells	15
C. Effect of carbon dioxide on respiration, fermentation and cell constituents	28
D. Cytological observations of the effect of carbon dioxide	31
<i>Chapter II. General Methods and Materials.</i>	<i>35</i>
A. Treatment of the material and methods of analysis.....	35
1. Experimental details	35
2. Succinate-succinic acid buffer (SS buffer)	37
3. Methods of analysis	41
B. Yeasts employed	45
1. Manufactured yeasts	45
2. Pretreated baker's yeasts	47
C. Effect of pretreatment on yeast. Characteristics of pretreated yeasts..	48
1. Cytological examination	49
2. Carbohydrate metabolism	52
3. Respiration and fermentation	56
4. Nitrogen metabolism	65
5. Phosphate distribution	67
6. Alterations in dry weight	71
7. Characteristics of pretreated yeasts	73
<i>Chapter III. Effect of Carbon Dioxide on the Metabolism and Cell Constituents</i>	<i>76</i>
A. Microscopical observations	76
B. Effect on growth	77
C. Effect on endogenous metabolism	79
D. Effect on free phosphate content	80
E. Effect on ammonium ion content	84
F. Effect on uptake of glucose	84
G. Effect on uptake of pyruvic acid	88
H. Summary of Chapter III	89

	Page
<i>Chapter IV. Determination of Acid-labile Bound Carbon Dioxide in the Yeasts</i>	90
A. Introduction	90
B. Method.....	91
1. Development of the method	91
2. Experiments with gaseous phase free from carbon dioxide	102
3. Preliminary experiments in the presence of carbon dioxide	103
4. Sources of error and correction of blank values	108
C. Determinations	113
1. Treatment of the measurements	113
2. Acid-labile bound carbon dioxide in baker's yeast. Effect of aerobic starvation, alteration of the pH value in the suspension medium, and irradiation	113
3. Carbon dioxide in fed yeast. Reversibility of starvation effect ..	118
4. Carbon dioxide in budding yeast	121
5. Corrected graphical mean curves	123
6. Effect of drying on the yeast	123
7. Influence of cell poisons on the capacity of the yeast for binding carbon dioxide	125
8. Influence of potassium and calcium ions	130
9. Carbon dioxide in mother yeasts	134
D. Summary of Chapter IV	136
<i>Chapter V. pH Determinations and Electrometric Titrations on Heat-fixed Yeasts.....</i>	138
A. Introduction	138
B. Method	142
C. Experimental Results	144
1. Determinations of pH	144
a) Dilution effect	145
b) Estimated pH values in baker's yeast and yeasts pretreated under various conditions	149
2. Electrometric titration curves	158
D. Summary of Chapter V	164
<i>Chapter VI. Discussion</i>	166
A. On the relation between the amount of acid-labile bound carbon dioxide and the pH in the yeast cell	166
B. Possibilities for the binding of carbon dioxide in yeast in forms other than bicarbonate	175
General Summary	186
References	191

Acknowledgements.

The present investigation was carried out in the Department of Cell Physiology at Wenner-Gren's Institute for Experimental Biology, University of Stockholm. To the Director of this Institute, Professor J. RUNNSTRÖM, I have pleasure in expressing my sincere gratitude for his encouragement and support throughout the six years during which I have been occupied in this work. Professor RUNNSTRÖM has initiated me into the physiological problems, given me ample opportunity for independent work, and placed laboratory accommodation and assistants at my disposal.

For valuable discussions, advice and criticism during the development of the investigation I cordially thank my friends Drs. P. E. LINDAHL, Å. ÖRSTRÖM, G. EHRENSVÄRD, MIGNON MALM and Mr. E. SPERBER.

I am indebted to Professor T. CASPERSSON for his kind co-operation in taking photographs of yeast cells in ultraviolet light with his well-known apparatus for this purpose.

For her never-failing interest and painstakingness, my thanks are due to my assistant, Miss ASTRID CARSJÖ, who has also drawn the figures in this thesis.

To my wife, KARIN BRANDT, I express my deep gratitude for valuable discussions in connection with this work and for her critical examination of the manuscript.

The translation of this paper into English has been carried out by Miss SOPHIE HELLMANN. The English text has been revised by Dr. D. CHEESMAN, especially with respect to the chemical terminology. For the great care they have displayed in this work, I wish to express my cordial thanks.

Finally, I take this opportunity of conveying my best thanks to Svenska Jästfabriks A.B., and particularly to their Chief Engineer, Mr. H. BRAHMER, for the helpfulness which they have always shown me.

This investigation has been performed with financial support from the Faculty of Science of Stockholm University, »Gustaf och Anna Retzius' fond för vetenskaplig forskning» of the Royal Swedish Academy of Sciences, »Stiftelsen Lars Hiertas Minne», and from funds provided by the Rockefeller Foundation to Wenner-Gren's Institute. A grant for the linguistic revision of the manuscript has been made by The British Council.

Stockholm, April 1945.

Knut Brandt.

General Introduction.

A problem of fundamental interest in cell physiology concerns the effect of changes in the carbon dioxide concentration in the living cell on the metabolism and the state of the protoplasm. Investigations performed in recent years have shown that carbon dioxide cannot exclusively be regarded as an end product in the metabolism of heterotrophic cells; it directly participates, indeed, in certain enzymic reactions within probably all cells. Carbon dioxide presumably also affects the physico-chemical properties of the protoplasm, which are intimately associated with the bicarbonate buffer system of the cell. Through the latter, carbon dioxide may indirectly influence the metabolism.

The problem of the effect of carbon dioxide on the function of the cell requires the study of the effect of probable variations in the acidity of the protoplasm. In the cells of most biological materials, the acidity of the protoplasm, *i. e.* the hydrogen ion concentration in the water phase of the cell content and hence the hydrogen ion concentration in the colloidal interfaces, generally corresponds to about pH 7 or somewhat less. The acidity of the protoplasm depends upon, among other factors, the carbon dioxide concentration in the cell and upon the ability of the protoplasm to bind carbon dioxide to alkali. It is of interest in this connection to examine whether enzymic reactions take place which counteract the changes in pH and whether such a regulating mechanism is dependent on the character of the metabolism. Furthermore, the study of the effect of carbon dioxide on living cells involves an examination of the direct influence on the state of the protoplasm exerted by changes in the concentrations of carbon dioxide and bicarbonate ions. A varying carbon dioxide concentration within the cell may arise through a change in the features and the intensity of the metabolism or through an alteration in the external physiological conditions. We may also put the unsolved question whether the effect of carbon dioxide

in intensity and quality in a clear manner from that of untreated yeast. The following description of methods and materials will include an account of certain observations concerning the features and the metabolism of the different yeasts. These observations are of importance in the consideration of the present studies and the problems arising in the course of the work.

A method for the determination of the quantity of acid-labile bound carbon dioxide in yeast has been developed on the basis of WARBURG's manometric method. Making use of this procedure, it has been possible to determine the quantity of carbon dioxide bound at different carbon dioxide and oxygen pressures in yeasts pretreated in various manners. In pursuance of these investigations, pH values were measured in the presence and absence of carbon dioxide on suspensions of yeasts, which were fixed by heating to 100° C. From these measurements and on the basis of observed dilution effects, changes in the pH of the protoplasm under different physiological conditions could be estimated. Finally, the experimental results are discussed with special regard to the relationship between estimated pH values and the quantity of acid-labile bound carbon dioxide, assuming that the latter consists of bicarbonate. As deviations appear between the values found and those calculated theoretically, various possibilities are examined regarding the binding of carbon dioxide other than as bicarbonate.

It is to be emphasized that calculations of the values for the acidity of the protoplasm from measurements of the quantity of acid-labile bound carbon dioxide within the intact cell can only be regarded as approximate. Data necessary for such calculations in the case of yeast cells are only available from measurements on serum, haemolysed blood corpuscles, or other injured cells. As carbon dioxide affects the metabolism of the cells and, consequently, a change of the latter obviously influences the carbon dioxide binding and the pH value of the protoplasm, a calculation will be valid only for those experimental conditions where the cells are in dynamic equilibrium with a gaseous phase of given composition. Up to the present, it cannot be stated conclusively whether, after a change in the physiological conditions, the dissociation constants, the activity coefficients, the solubility of carbon dioxide in the protoplasm, and the carbon dioxide concentration in the intact cell (despite the same carbon dioxide content in the gaseous phase) are constant. This uncertainty involves a

change in the properties of the cell and its metabolism in the course of the measurements and may be assumed to be especially significant under intentionally changed physiological conditions. As, however, changes in the physiological conditions are necessary to provide an insight into their effect on the properties of the protoplasm, this uncertainty cannot be avoided. This is a difficulty which must always be kept in mind during work on living material.

CHAPTER I.

The Effect of Carbon Dioxide on Living Cells and its Significance in Biological Processes. A Review.

A. The Importance of Carbon Dioxide for the Growth of Micro-organisms.

Carbon dioxide is of essential importance for the normal functioning of living cells of the most different types. Investigations in this field have been made by studying the effect of carbon dioxide on the metabolism and the protoplasm, either during treatment of the cells with carbon dioxide under different carbon dioxide pressures or by removing the carbon dioxide formed during metabolism as completely as possible from the environment of the cells. Before 1935, the active participation of carbon dioxide in metabolism was known only in assimilation processes occurring in autotrophic organisms, *i. e.* those organisms which synthesize their cell material exclusively from inorganic compounds where carbon dioxide is the sole source of carbon. However, numerous earlier investigations had shown that even the heterotrophic cells, which require for their growth organically bound carbon, are greatly dependent on and affected by carbon dioxide.

At the end of the 19th century, it was already well known that high concentrations of carbon dioxide frequently have an injurious effect on various micro-organisms. This effect, however, is astonishingly small in some cases. Thus, it was found that some bacteria, and even yeast cells, in the resting state could stand a carbon dioxide pressure of about 40 atmospheres for more than one day without being killed (cf. LARSON, HARTZELL and DIEHL 1918). Nevertheless, the growth of, for example, micro-organisms is already restricted at a much lower carbon dioxide pressure than a

fractional part of atmospheric pressure. Earlier literature concerning the effect of carbon dioxide on bacteria has been reviewed by VALLEY (1928); see also KNIGHT's index (1936).

When isolating *Brucella abortus*, NOWAK (1908) observed that its development was favoured when the cultures grew in a common gaseous phase together with the aerobe *Bact. subtilis*. This phenomenon was attributed to a reduction of the oxygen pressure. Later, however, the same effect could be obtained by adding carbon dioxide to the air; the furtherance of the growth should thus be attributed to carbon dioxide and not to reduced oxygen pressure. This was pointed out by, among others, SMITH (1924), MASON (1926), McALPINE and SLANETZ (1928), and WILSON (1931), who obtained an optimum growth for some strains of *Br. abortus* when adding 5--10 % carbon dioxide to the gaseous phase. In the cultivation of meningococci (COHEN and FLEMING 1918) and gonococci (CHARIN 1918), a carbon dioxide concentration of c. 10 % yields optimum growth (in some cases, the presence of even up to 30 % carbon dioxide brought about good growth in meningococci cultivations). For the more recent literature, the reader should consult SHAUGHNESSY (1939). The effect of carbon dioxide on the growth of bacteria during variations in the oxygen pressure and the composition of the substrate has been studied in greater detail by LAGONI (1939) and by WILLIAMS (1939).

WHERRY and ENIX (1918) were the first to show that the growth of heterotrophic bacteria was suppressed by the removal of carbon dioxide from the culture medium. In their experiments on tubercle bacteria they found no increase in growth when carbon dioxide was absorbed by a solution of barium or sodium hydroxide. It may be mentioned here that for the growth of these bacteria the optimum carbon dioxide concentration in the gaseous phase is only c. 2.5 % when the pH value of the substrate is 7.4 (DAVIES 1940). SMITH (1924) stated that the presence of carbon dioxide was indispensable to the growth of *Br. abortus*. Furthermore, ROCKWELL and co-workers (see ROCKWELL and HIGHBERGER 1927), in a series of investigations, demonstrated that carbon dioxide favoured the growth of various aerobic and facultatively anaerobic bacteria, the latter even under anaerobic conditions. Growth was impeded even in these organisms if carbon dioxide was removed by incubating plate-cultures over alkaline solutions. Investigations by VALLEY and RETTGER (1926, 1927), who studied more than 100 different types of bacteria, confirmed that the

presence of carbon dioxide was a necessary condition for the development of both anaerobic and aerobic bacteria.

All investigations mentioned above have been carried out on plate-cultures. Experiments performed by WALKER (1932), WINSLOW, WALKER and SUTERMEISTER (1932), and later by GLADSTONE, FILDES and RICHARDSON (1935), showed that even for cultures in liquid media the indispensability of carbon dioxide for the growth of bacteria could be proved by aerating with carbon dioxide-free air. Removal of carbon dioxide did not, however, cause the death of the cells.

In their experiments on moulds (*Mucor*) and yeast (*Saccharomyces*), ROCKWELL and HIGHBERGER (1927) were able to show that growth was checked or prevented if the vigorous production of carbon dioxide obtained in the growth metabolism of these fungi was restrained and the carbon dioxide produced was kept below a certain threshold value by appropriate experimental conditions as, for example, low substrate concentration, small inoculation amounts, and proper salt environments. On the basis of their own results and those of other workers, these investigators assumed that carbon dioxide was involved directly in the growth of all bacteria, yeasts and moulds, and the gas was used as a source of carbon.

RIPPEL and BORTELS (1927) demonstrated that the presence of carbon dioxide was necessary for the development of the spores of *Aspergillus niger*. Later, HEILMANN (1929) and RIPPEL and HEILMANN (1930) confirmed this finding and pointed out the significance of small concentrations of carbon dioxide in the development of spores of *Penicillium*, *Rhizopus*, *Botrytis* and other fungi. Furthermore, PLATZ, DURRELL and HOWE (1927) examined the growth of the chlamydospores of *Ustilago Zeae*. They found that up to 15 % carbon dioxide in the gaseous phase promoted the development, while this was impeded in the absence of carbon dioxide. Recently, GOLDING (1940) investigated the effect of various carbon dioxide concentrations on the growth of *Penicillium roqueforti* in air and found that, just as in the cultivation of bacteria, growth was promoted by concentrations of carbon dioxide in the gaseous phase up to 20—30 %, while it was impeded by high concentrations (cf. earlier investigations by BROWN 1922 on various fungi).

The carbon dioxide requirements of micro-organisms in their growth processes were demonstrated not only in experiments on

bacteria and moulds, yeasts and other fungi, but also in tests on protozoa. Thus RAHN (1941), in his experiments in hanging drops, reported that the protozoa *Polystomella* and *Astasia* did not reproduce if carbon dioxide was removed by addition of potassium hydroxide to the water on the bottom of the moist chamber.

B. Carbon Dioxide Fixation and Reduction in Autotrophic and Heterotrophic Cells.

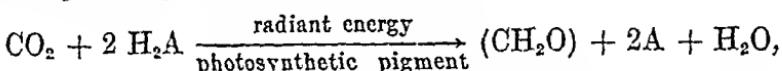
Investigations performed in recent years have contributed essentially to the understanding of the carbon dioxide requirements of different organisms. By the use of carbon dioxide marked with the radioactive isotope ^{14}C (half life = 21 min.) or the stable heavy isotope ^{13}C , it could be proved that carbon dioxide participated in enzymic reactions even within heterotrophic cells. Both in micro-organisms and in cells of animal tissue a fixation of carbon dioxide takes place, which is followed by an enzymic reduction of carbon dioxide. KLUYVER (1939) has reviewed the earlier literature regarding the part played by carbon dioxide in the metabolism of autotrophic and heterotrophic cells. He points out that this gas probably plays a general and significant rôle in the processes occurring within all cells. KLUYVER concludes his review on the effect of carbon dioxide as follows: "Während PLINIUS sie (die Kohlensäure) bald nach Anfang unserer Zeitrechnung als 'spiritus letalis' deutete, liegt, angesichts der ans Licht getretenen Unentbehrlichkeit dieses Gases für alles was lebt, vielmehr Grund vor, ihr den Namen zu schenken 'spiritus vitalis'." In a survey entitled "Spiritus Vitalis", FOSTER, CARSON and RUBEN (1941) expanded KLUYVER's conception of the general importance of carbon dioxide to all cells. Their review was based on recent experiences of carbon dioxide metabolism in autotrophs and heterotrophs, obtained with the help of the carbon isotopes mentioned above. Further summaries concerning the latest results on fixation and reduction of carbon dioxide, especially in heterotrophic cells, have been submitted by EVANS (1942), WERKMAN and WOOD (1942), and KREBS (1943). In the following discussion, it is intended to give a short review of the participation of carbon dioxide in the metabolism of autotrophs and especially in that of heterotrophs. This account seems to be justified in view of the importance of carbon dioxide "assimilation" in the interpretation

of the author's observations regarding the effect of carbon dioxide on yeast metabolism. More detailed information may be gained from the papers cited above.

Photosynthesis.

It is well known that green plants and autotrophic bacteria are able to utilize carbon dioxide in their metabolism as their only source of carbon. These organisms reduce carbon dioxide by utilizing either light energy or energy obtained through a chemical reaction. Autotrophic cells are characterized as photosynthetic or chemosynthetic in accordance with the type of energy source exploited by them.

According to VAN NIEL (1931), the photosynthetic processes may be generally formulated as follows:

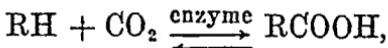


where H_2A denotes the hydrogen donor. In plant cells containing chlorophyll, water serves as a hydrogen donor. This relationship, originally suggested by KLUYVER and DONKER (1926), was later proved by RUBEN, RANDALL, KAMEN and HYDE (1941), who worked with water containing the heavy oxygen isotope ^{18}O . In the green sulphur bacteria (*Thiobacteriales*) containing a pigment similar to plant chlorophyll, hydrogen sulphide acts as a hydrogen donor. Thus, instead of oxygen, free sulphur is produced which is not further oxidized. In the photosynthetic assimilation of carbon dioxide by the purple sulphur bacteria (*Thiorhodaceae*) discovered by ENGELMANN (1883) and subsequently studied by WINOGRADSKY and MOLISCH, hydrogen sulphide also serves as a hydrogen donor. These bacteria, however, also oxidize sulphite, thiosulphate, and free sulphur (see, *inter alia*, VAN NIEL 1931) with the simultaneous photoreduction of carbon dioxide. Under completely anaerobic conditions, even organic substances and, in certain cases, molecular hydrogen may serve as hydrogen donors in the purple sulphur bacteria (ROELOFSEN 1935).

The non-sulphur purple bacteria (*Athiorhodaceae*) discovered by MOLISCH (1907) occupy an intermediate position between autotrophic and heterotrophic bacteria. The presence of organic substances, such as aliphatic acids, is a condition for the photosynthetic reduction of carbon dioxide in these bacteria (VAN NIEL 1931). In this connection it must be emphasized that the organic

compounds may serve as hydrogen donors without a break-down of the carbon chain in the oxidizable substrate taking place (FOSTER 1940). For further details of bacterial photosynthesis the reader is referred to VAN NIEL's (1941) comprehensive review.

In recent years, the application of tracer carbon has extended our knowledge of the photosynthetic processes. Experiments by RUBEN, KAMEN, HASSID and DE VAULT (1939), followed by a series of investigations by RUBEN and his co-workers (cf. RUBEN and KAMEN 1940 a), have shown that the photosynthetic reduction of carbon dioxide in green plants is preceded by a fixation of carbon dioxide in the cell with the formation of a carboxyl group (cf. SMITH and COWIE 1941). This reaction is reversible and occurs at the same rate in the dark and in the light; it was denoted as "the dark reaction" and may provisionally be written as follows:



where RH probably represents a reduced enzyme or a prosthetic group containing an aldehyde group. According to measurements performed up to the present (with *Chlorella pyrenoidosa*), the intermediate product (RCOOH) has a molecular weight of about 1 000. According to RUBEN's (1943) hypothesis, RH is first phosphorylated by an energy-rich phosphate donor as, for example, adenosine triphosphate (cf. LIPMANN 1941 and KALCKAR 1942). Subsequently, carbon dioxide is fixed, while phosphate is split off. Assuming the carbon dioxide to be fixed to an aldehyde group, the compound produced may be written as R·CO·COOH. This is reduced enzymically to R·CHOH·COOH which, probably after further phosphorylation, may be reduced to R·CHOH·CHO. Through another cycle of phosphorylation, carboxylation, and reduction, it should be possible to repeat the reaction. The photochemical reaction is assumed to be responsible only for the production of special hydrogen donors originating in the photodecomposition of water (VAN NIEL 1941).

It should be emphasized here that the above-mentioned dark reaction of photosynthesis is of fundamental interest, since a similar fixation of carbon dioxide by carboxylation seems also to occur in chemo-autotrophic and heterotrophic cells. The ability of photosynthetic cells to bind and to reduce carbon dioxide may presumably be considered a special case of a generally occurring biological fixation and reduction of carbon dioxide. In non-

photosynthetic cells, the energy necessary for the reduction originates exclusively in the oxidation of different organic and inorganic substances.

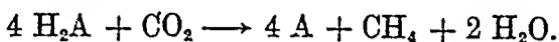
Carbon Dioxide Assimilation in Chemo-autotrophic Bacteria.

The utilization of carbon dioxide as the sole source of carbon in metabolism, even in the absence of light, has been known for very long. Bacteria of a chemo-autotrophic character were first described by HEREAUS (1886), who studied certain nitrifying soil bacteria. He assumed that these bacteria were capable of synthesizing their cell material from inorganic salts and carbon dioxide. A series of investigations performed during the following years, primarily by WINOGRADSKY, confirmed HEREAUS' observations and showed that, in the presence of carbon dioxide, colourless sulphur bacteria and thread-formed iron bacteria were also able to grow in exclusively inorganic media in the dark and under aerobic conditions. The energy indispensable for the reduction of carbon dioxide in these chemo-autotrophic cells was found to originate in the oxidation of single inorganic compounds. As examples, mention may be made of the oxidation in nitrifying bacteria of ammonia to nitrite (*Nitrosomonas*) and of nitrite to nitrate (*Nitrobacter*) and, finally, of the oxidation in colourless sulphur bacteria of hydrogen sulphide to free sulphur (*Beggiatoa*) and of free sulphur to sulphate (*Thiobacillus thio-oxidans*). *Didymohelix* and *Crenothrix* oxidize ferrous and manganous compounds respectively. Further examples are the oxidation of molecular hydrogen by *Carboxydomonas oligocarbophilia* and that of methane by *Methanomonas methanica* with the simultaneous reduction of carbon dioxide. VOGLER (1942) has studied more thoroughly the fixation of carbon dioxide in the dark by *Thiobacillus*. He has shown that carbon dioxide can be fixed under aerobic and anaerobic conditions and that the presence of sulphur does not influence the somewhat limited carbon dioxide fixation obtained under anaerobic conditions.

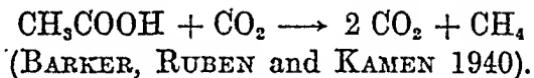
Methane Fermentation.

As early as in the beginning of this century, it was observed that, in certain obligate anaerobic bacteria, carbon dioxide was reduced in the dark. It was not possible, however, to relate this phenomenon with a simultaneous oxidation of inorganic com-

pounds, as may be done in the case of the previously mentioned chemo-autotrophic bacteria. Experiments performed, *inter alia*, by HOPPE-SEYLER, OMELIANSKI, and SÖHNGEN on methane-fermenting bacteria showed that different organic compounds could be converted in such a way that carbon was converted almost quantitatively into carbon dioxide and methane (cf. SÖHNGEN 1906). VAN NIEL (cited in BARKER 1936 a) assumed that, in the fermentation of methane, the organic substrate acted as a hydrogen donor in the hydrogenation of simultaneously present carbon dioxide. This theory was later completely confirmed, chiefly by BARKER's investigations (1936 a, 1936 b, 1941). For the general course of methane fermentation this worker (1936 a) suggested the following scheme:



H_2A represents an oxidizable organic compound serving as hydrogen donor. Thus, in the presence of carbon dioxide, primary and secondary aliphatic alcohols are oxidized to the corresponding fatty acids and ketones by *Methanobacterium Omelianskii*. Acetic acid may possibly be further oxidized to carbon dioxide by *Methanosarcina methanica* according to the equation



BARKER (1941), applying the tracer method, showed that in the first mentioned bacteria the carbon from ethanol and carbon dioxide was to a certain extent incorporated with the cell material. Although methane bacteria reduce the greater part of the carbon dioxide to methane, examples exist of its fixation by and incorporation with the constituents of these cells. According to the observations mentioned above, the fermentation of methane may be interpreted as a reduction of carbonate, in analogy with the well-known bacterial processes resulting in the reduction of sulphate and nitrate to hydrogen sulphide and ammonia respectively (cf. BARKER 1936 a). The mechanism of the reduction of carbon dioxide to methane, however, is still unknown. According to BARKER's (1941) investigations, the intermediate product is presumably not formic acid (cf. STEPHENSON and STICKLAND 1933). Formate can replace neither ethanol as a hydrogen donor nor carbon dioxide as a hydrogen acceptor.

Miscellaneous Fixation Reactions.

The formation of formic acid by the reduction of carbon dioxide with molecular hydrogen was shown in experiments by WOODS (1936) on *Escherichia coli*. Earlier, STEPHENSON and STICKLAND (1932) and YUDKIN (1932) described an adaptive enzyme, formic hydrogen-lyase, in, *inter alia*, *E. coli* and *Bact. lactis aerogenes*, by which formic acid was split into carbon dioxide and hydrogen. Experiments by Woods indicated that this reaction was reversible and resulted in the formation of formic acid from $\text{CO}_2 + \text{H}_2$.

As far as is known at present, both formic acid and methane are metabolic end products which should not be regarded as "assimilation products", since they do not lead to an incorporation of carbon with the cell structure. Consequently also the participation of carbon dioxide in the formation of urea should not be considered an assimilation process. According to the deductions of KREBS and HENSELEIT (1932), later confirmed by RITTENBERG and WAELSCH (1940) and by EVANS and SLOTIN (1940 b), who worked with the carbon isotopes ^{13}C and ^{14}C respectively, carbon dioxide and ammonia in the liver are bound to ornithine to form citrulline which, together with ammonia, yields arginine. This compound is subsequently broken down by arginase into urea and ornithine. Carbon dioxide, however, may be of direct importance in the synthesis of creatine, as in arginine the guanidino group, originating from a fixation of carbon dioxide, is converted together with glycine to glucoccyamine. This latter may be supposed to be an intermediate product in the synthesis of creatine (see KREBS 1943).

In 1936 and 1940, WIERINGA (cited in WERKMAN and WOOD 1942) demonstrated the participation of carbon dioxide also in the formation of acetic acid. In experiments with an anaerobic spore-forming bacterium belonging to the clostridium group (*Clostridium aceticum*) a formation of acetic acid was obtained from $2 \text{CO}_2 + 4 \text{H}_2$. By virtue of its ability to live on an inorganic medium in the presence of only traces of some unknown organic substance — presumably of coenzymic character — the said bacterium occupies an intermediate position between the chemoautotrophic and the heterotrophic organisms. BARKER, RUBEN and BECK (1940) worked experimentally on the *Cl. acidi urici* isolated by BARKER, which exhibits a more clearly heterotrophic character than *Cl. aceticum*, a fact which appears in its capacity

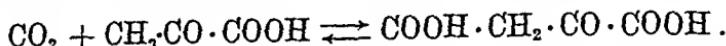
to reduce carbon dioxide under anaerobic conditions during the oxidation of uric acid and other purines. This species also formed acetic acid, which proved to contain carbon dioxide-carbon (¹⁴C) in both the methyl and the carboxyl groups. The mechanism of coupling two carbon dioxide molecules to acetic acid, however, is still quite unknown. It may be mentioned here that SLADE, WOOD, NIER, HEMINGWAY and WERKMAN (1942), in experiments with *Cl. welchii*, observed a formation of acetic acid. In this case the acetic acid contained carbon dioxide-carbon in the carboxyl group only. Here acetic acid seems to occur by the cleavage of a primarily formed C₄-dicarboxylic acid. Furthermore, BARKER (1944) has shown that, in the anaerobic metabolism of glucose and xylose by *Cl. thermo-aceticum*, apart from the cell material and traces of carbon dioxide, only acetic acid is formed. Estimations of the amount of acetic acid formed during the metabolism of the said substrate, and of carbon dioxide formed during the turnover of pyruvic acid, indicate that in this bacterium also carbon dioxide is converted into acetic acid.

Carboxylation of Pyruvic Acid. Formation of Dicarboxylic Acids and Related Compounds.

Investigations of Micro-organisms.

In the introduction to this chapter, some observations were discussed regarding the inhibition of the growth of different micro-organisms following the removal of carbon dioxide from their environment. These observations indicate that carbon dioxide participates actively in the metabolism proper even within the strictly heterotrophic cells. Powerful support for such an interpretation was given by WOOD and WERKMAN (1935, 1936), who observed that in the fermentation of glycerol with propionic acid bacteria the end products, chiefly propionic acid and succinic acid, contained more carbon than corresponded to the added glycerol. The organically bound carbon excess, however, could be related to a loss of calcium carbonate which had been added in order to neutralize the acids formed. Further investigations (WOOD and WERKMAN 1938) showed that, in the fermentation of glycerol, the carbonate consumed (or CO₂) was equivalent to the amount of succinic acid formed. (In the absence of carbon dioxide, however, no succinic acid was formed.) These workers assumed that succinic acid was formed, via oxaloacetic acid, by a fixation of

carbon dioxide to pyruvic acid, the occurrence of which in the fermentation of propionic acid had been earlier demonstrated:



A direct proof of carbon dioxide fixation during the formation of succinic acid in propionic acid bacteria was given later by CARSON and RUBEN (1940) and by WOOD, WERKMAN, HEMINGWAY and NIER (1940, 1941 a, 1941 b). The latter, using the tracer method, observed that in succinic acid the carbon dioxide-carbon added was recovered in the carboxyl-carbon. Labelled carbon dioxide was also shown in the propionic acid formed. (Possible explanations of these reactions were discussed by KREBS (1943) to whom the reader is referred for further details.) A formation of the very unstable oxaloacetic acid in the initial reaction, as assumed by WOOD and WERKMAN, could not be detected. However, KREBS and EGGLESTON's (1940, 1941) investigations on other material with regard to the metabolism of pyruvic acid lend strong support to the existence of this reaction (cf. below).

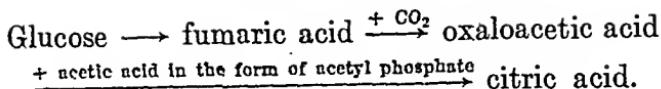
The active participation of carbon dioxide in the formation of succinic acid in heterotrophic cells other than propionic acid bacteria was indicated by an observation of ELSDEN (1938). This author found that the rate at which succinic acid was formed by *Escherichia coli* was proportional to the carbon dioxide pressure. Further investigations with labelled carbon have shown that in all cells investigated, *i. e.* both in micro-organisms and in animal cells, carbon dioxide can be fixed by and incorporated with organic material within the cell. RUBEN and KAMEN (1940 b) found that small amounts of carbon dioxide were bound in the dark by *E. coli*, yeast, minced plant (barley) roots, minced rat liver tissue, and *Chlorella* cells. It was later confirmed that carbon dioxide was also fixed by filamentous fungi, LEBEDEW juice from yeast, *Acetobacter suboxydans*, and *Staphylococcus faecalis* (RUBEN, CARSON, KAMEN and FOSTER, unpublished; cf. FOSTER, CARSON and RUBEN 1941).

KLEINZELLER's (1941) experiments on yeast revealed that, if glucose was metabolized in the presence of carbon dioxide and sodium bicarbonate, both under aerobic and anaerobic conditions, succinic acid was formed. It was not, however, obtained either in the absence of carbon dioxide or sodium bicarbonate or during the growth of the yeast. In the presence of glucose, the yield of succinic acid was clearly increased when oxaloacetate, malate or

aspartate was added. With additional evidence from ELSDEN, RUBEN and their co-workers' observations and from KREBS and EGGLESTON's (1940) investigations in the metabolism of pyruvic acid in animal tissue, KLEINZELLER assumed that in yeast also succinic acid was formed from oxaloacetic acid arising from the carboxylation of pyruvic acid.

This hypothesis in its more general application was further supported by the experiments of WOOD, WERKMAN, HEMINGWAY and NIER (1941 a, 1941 b), NISHINA, ENDO and NAKAYAMA (1941, cited in WERKMAN and WOOD 1942), and SLADE, WOOD, NIER, HEMINGWAY and WERKMAN (1942) who all applied the tracer method. These investigators showed that during the metabolism of glucose or, in certain cases, pyruvic acid or citric acid, carbon dioxide was assimilated in different micro-organisms. In all cases, labelled carbon dioxide was recovered in, *inter alia*, the succinic acid formed (experiments on *Propionibacterium pentosaceum*, *Escherichia coli*, *Aerobacter indologenes*, *Streptococcus "paracitrovorus"*, *Proteus vulgaris*, and *Staphylococcus candidus*). Isotopic carbon was also observed in lactic acid from these organisms, except for the two first-named (see review in KREBS 1943). Moreover, NISHINA and co-workers studied the fermentation of pyruvic acid in *E. coli* in the presence of labelled carbon dioxide; they recovered the labelled carbon in the malic and the fumaric acids formed. This observation commands special interest, since these acids were earlier assumed to be the intermediate products in the formation of succinic acid from the hypothetical oxaloacetic acid in *Propionibacterium* and also in *Staphylococcus* (the latter studied by SMYTH 1940).

In experiments on moulds, FOSTER, CARSON, RUBEN and KAMEN (1941), using ^{14}C as a tracer, found that when they cultivated *Rhizopus nigricans* in a glucose solution in the presence of carbon dioxide, both under aerobic and anaerobic conditions, fumaric acid was formed which contained labelled carbon dioxide-carbon in the carboxyl groups. Even in the acid-insoluble cell material (probably protein), ^{14}C was recovered. Experiments on the metabolism of glucose in *Aspergillus niger* yielded citric acid which also contained the tracer carbon in the carboxyl groups. For purposes of comparison with probable reactions during the fixation of carbon dioxide in certain animal cells (cf. below), it may be noted that KREBS (1943) proposed and discussed the following equation for the synthesis of citric acid in moulds:



A fixation of carbon dioxide has also been demonstrated in protozoa. Thus, SEARLE and REINER (1940, 1941) showed that under anaerobic conditions *Trypanosoma lewisi* forms succinic acid, lactic acid, pyruvic acid, and acetic acid during the fermentation of glucose. This reaction occurs, however, only if the medium contains bicarbonate. Just as in the case of propionic acid bacteria, carbon dioxide is here assimilated in an amount equivalent to the succinic acid formed. Under aerobic conditions succinate is formed, but no carbon dioxide is consumed. When glycerol is used as substrate, no succinate is formed. Furthermore, VAN NIEL, THOMAS, RUBEN and KAMEN (1942), with the aid of ^{14}C as a tracer, found that *Tetrahymena geleii* (holotrichous ciliate) under anaerobic fermentation of sugar forms, *inter alia*, succinic acid containing ^{14}C in the carboxyl groups.

Investigations of Animal Cells.

The assimilation of small amounts of carbon dioxide in animal cells was reported by RUBEN and KAMEN (1940 b) in their previously mentioned observation that labelled carbon dioxide was fixed by minced rat liver. Experiments performed by KREBS and EGGLESTON (1940), EVANS and SLOTIN (1940 a, 1941) and WOOD, WERKMAN, HEMINGWAY and NIER (1941 c, 1942) made it clear that the rôle of carbon dioxide in liver metabolism was associated with the oxidative dissimilation of pyruvic acid, taking place according to the modified "citric acid cycle" (also denoted as the tricarboxylic acid cycle). The probable course of this reaction is illustrated in Fig. 1, which represents a preliminary scheme taken from the paper of WERKMAN and WOOD (1942). In muscle tissue, malonate checks the oxidation of succinate to fumarate, and thus the formation of oxaloacetate derived from these acids; pyruvic acid reacts with the latter when coupled into the cycle. In contrast to this behaviour, it was found that in liver, despite the addition of malonate, an oxidation of pyruvic acid took place. This may be explained by the fact that, in the liver, a formation of oxaloacetic acid may also occur by the fixation of carbon dioxide to pyruvic acid.

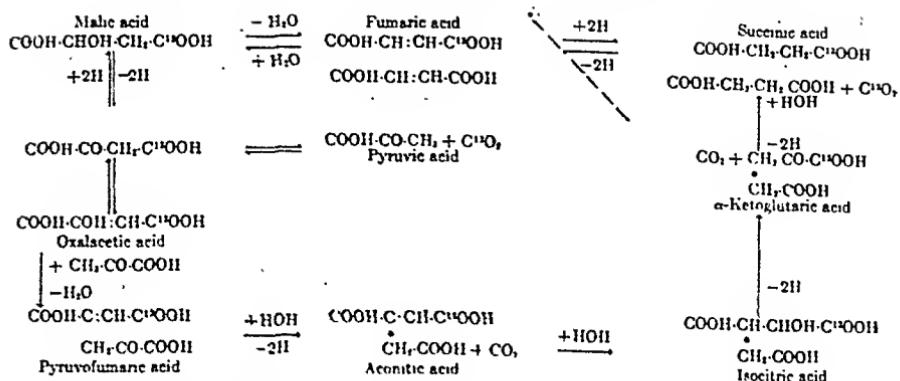
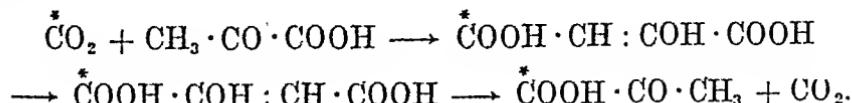


Fig. 1. Dissimilation of pyruvate by pigeon liver (from WERKMAN and WOOD 1942).

Applying ^{14}C as tracer carbon, EVANS and SLOTIN showed that during the metabolism of pyruvic acid in pigeon liver, carbon dioxide was fixed and was recovered, *inter alia*, in the α -ketoglutaric acid formed. The labelled carbon atom was fixed to the carboxyl group adjacent to the α -keto group. Further experiments revealed that the radioactive carbon was probably also bound to the carboxyl groups in α -amino acids. Moreover, in experiments with pigeon liver, WOOD and co-workers observed that in the presence of pyruvic acid and carbon dioxide (labelled with ^{14}C) carbon dioxide-carbon could be recovered from fumaric, malic and succinic acids and, in smaller quantities, from lactic acid. Here, also the fixed carbon atom was detected in the carboxyl groups of all acids.

The occurrence of lactic acid containing isotopic carbon in the liver and in certain heterotrophic bacteria (cf. above) can presumably be explained by the assumption that enol-oxaloacetic acid is first formed in which the hydrogen and hydroxyl in the α and β positions change places according to the following equation:



Decarboxylation yields pyruvic acid which is subsequently reduced to lactic acid. This latter contains labelled carbon dioxide-carbon in the carboxyl groups.

In their investigations of starving rats, SOLOMON, VENNESLAND, KLEMPERER, BUCHANAN and HASTINGS (1941) have proved that

labelled carbon dioxide is found in the liver glycogen if radioactive sodium bicarbonate is injected and lactate is simultaneously administered by stomach tube. In this reaction, lactic acid is possibly oxidized to pyruvic acid which, together with carbon dioxide, yields dicarboxylic acids. It may be assumed that phospho-enol-oxaloacetic acid is formed by a phosphorylation process which probably occurs in connection with an oxidation of fumarate. After decarboxylation, this phosphorylated compound would yield phosphopyruvic acid. Subsequently, a resynthesis is conceivable via the well-known intermediate products between glycogen and phosphopyruvic acid (cf. also LIPMANN 1941). At a later date, the participation of carbon dioxide in the synthesis of glycogen has been demonstrated in experiments on the liver tissue *in vitro* (VENNESLAND, SOLOMON, BUCHANAN and HASTINGS 1942, and BUCHANAN, HASTINGS and NESBETT 1942). Experiments with tracer carbon have also revealed the participation of carbon dioxide in the formation of glycogen within the muscle (HASTINGS and KISTIAKOWSKY 1940, and LORBER, HEMINGWAY and NIER 1943).

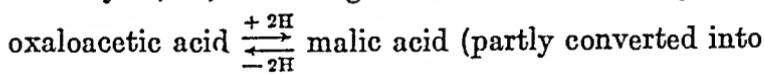
Investigations in Cell-free Systems.

In recent years, the investigation of carbon dioxide fixation by tracer methods has been extended to the study of cell-free enzyme systems. These investigations will probably contribute much to the understanding of the still very hypothetical reactions occurring in the fixation and reduction of carbon dioxide. Using an enzyme preparation from *Escherichia coli*, KALNITSKY and WERKMAN (1942) succeeded in demonstrating that, under anaerobic conditions, a fixation of carbon dioxide was obtained in the presence of pyruvic acid, one of the products being succinate containing labelled carbon (^{14}C) in the carboxyl groups. KRAMPITZ and WERKMAN (1941), and KRAMPITZ, WOOD and WERKMAN (1943) found a cocarboxylase-free enzyme system from *Micrococcus lysodeikticus* which, in the presence of Mg^{++} , decarboxylated oxaloacetic acid to pyruvic acid. According to observations in the presence of $^{14}\text{CO}_2$, this enzyme system is probably responsible also for the reversible reaction. These are the first experiments which indicate directly that oxaloacetic acid is, as supposed above, the product of the fixation of carbon dioxide.

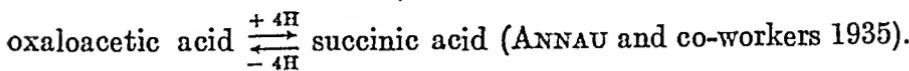
EVANS, SLOTIN and VENNESLAND's (1942) investigations have further shown that carbon dioxide is also fixed in cell-free extracts

of pigeon liver. Both under aerobic and anaerobic conditions, carbon dioxide is fixed in the presence of fumaric and pyruvic acids; with increasing concentration of pyruvic acid an increasing carbon dioxide consumption has been observed. From further experiments, EVANS, VENNESLAND and SLOTIN (1943) concluded that the extracted enzyme system possibly corresponded to that obtained by KRAMPITZ and co-workers. These experiments supported the view that carbon dioxide was fixed by pyruvic acid with the formation of oxaloacetic acid or its derivatives.

The above observations concerning the fixation and reduction of carbon dioxide within the typically heterotrophic cells make it clear that, in practically all cases investigated, the carbon dioxide may be traced to, *inter alia*, the carboxyl groups of dicarboxylic acids. It is found primarily in succinic acid, also in fumaric and malic acids and, finally, in animal cells, in α -ketoglutaric acid. The supposition that carbon dioxide reacts with pyruvic acid with the formation of oxaloacetic acid in most biological materials agrees well with the occurrence of labelled carbon dioxide-carbon within the said dicarboxylic acids. This may also explain the existence of carbon originating from carbon dioxide in lactic acid, pyruvic acid and propionic acid. The presence of carbon dioxide-carbon in citric acid, glycogen, and certain amino acids may also be very reasonably ascribed to reactions with acids derived from oxaloacetic acid in the modified citric acid cycle. The formation of dicarboxylic acids from carbon dioxide and pyruvic acid tends to favour the view that carbon dioxide is of essential significance in oxido-reductive processes. Both in animal cells and in numerous micro-organisms, these latter processes occur with the aid of the citric acid cycle, or, according to SZENT-GYÖRGYI's system,



fumaric acid), or, in certain cases,



This assumption is strongly supported by the investigations of HES (1938), which show that carbon dioxide probably plays an extremely important part in the reducing systems in *Escherichia coli*, *Bact. prodigiosum*, and the fungi *Aspergillus*, *Saccharomyces* and *Prototheca*. Applying the methylene blue reduction method of THUNBERG, HES found that the removal of carbon dioxide from the washed cell suspensions inhibited or slowed down the reduc-

tion of methylene blue, while addition of carbon dioxide catalysed this reaction.

Since, in the present work, yeast has been used as experimental material, it is of immediate interest to clear up the question whether carbon dioxide plays the same rôle in the oxido-reductive processes of yeast cells during the metabolism. ROCKWELL and HIGHERGER (1927) showed that carbon dioxide was indispensable to the growth of yeast. RUBEN and KAMEN (1940 b) found a fixation of carbon dioxide in yeast. Both KLEINZELLER's (1941) observations that carbon dioxide can participate in the formation of succinic acid, and HES's result that carbon dioxide plays an important part in dehydrogenation reactions in yeast support the theory that carbon dioxide plays an essential rôle in oxido-reductive processes. This seems to hold true also for yeast cells, especially if the observations made in experiments on various other materials are considered.

C. Effect of Carbon Dioxide on Respiration, Fermentation and Cell Constituents.

The observations described so far have only concerned the effect of carbon dioxide and its necessity for the normal development and growth of micro-organisms, together with the participation of carbon dioxide in certain enzymic reactions in autotrophic and heterotrophic cells. Apart from these investigations, little work has been carried out where the effects of carbon dioxide on the metabolism and on certain cell constituents are studied. Such work alone provides data regarding the reversible effect of carbon dioxide on the respiration and the pH in the objects investigated, as well as the different behaviours of carbon dioxide under aerobic and anaerobic conditions. The data, which have been obtained from experiments on both animal cells and plant material, are of special interest in the subsequent discussion, since they agree well with and help to elucidate the facts accumulated in the author's own investigations on yeast (Chapters III—V).

In his experiments on the fertilized sea-urchin egg, WARBURG (1910) could not detect any difference in the magnitude of the respiration of these cells when they were kept in air or exposed to a carbon dioxide pressure of 15 mm. Hg. Employing an improved method, Root (1930) found, however, that the rate of oxygen consumption of the fertilized *Arbacia* egg was depressed even by

very small concentrations of carbon dioxide in the gaseous phase. Above 30 mm. Hg, a further increase in the carbon dioxide tension (investigated up to 180 mm. Hg) has a relatively slight effect on the respiration. In experiments on *Paramaecium*, however, an optimum oxygen consumption was reached at a carbon dioxide pressure of c. 50 mm. Hg. LASER and ROTHSCHILD (1940) report that in the manometric determination of the respiration of the fertilized *Psammechinus* egg, reduced respiration is obtained if carbon dioxide is removed by absorption in potassium hydroxide.

Recently, CRAIG and BEECHER (1943) found in experiments on retina with constant pH in the medium that both respiration and glycolysis were almost doubled when the carbon dioxide concentration was raised from 1 to 5 %. Further increase in the carbon dioxide concentration from 5 to 20 %, however, considerably impeded the respiration without affecting the glycolysis. The presence of 20 % carbon dioxide did not depress the metabolism of cerebral cortex or *medulla oblongata*.

Numerous investigators have studied the effect of carbon dioxide upon fruits and other vegetables during storage. Moderate concentrations of carbon dioxide have proved to favour the preservation of these goods and to impede the ripening processes in different fruits. Concentrations higher than 50 % carbon dioxide in the gaseous phase, however, frequently appeared to be injurious. In connection with investigations of this kind, THORNTON (1933 a) examined the effect of carbon dioxide on the uptake of oxygen by fruits and vegetables. He found that storage in an atmosphere of 20 % oxygen and 10—30 % carbon dioxide often slightly impeded the oxygen consumption which, however, was increased by higher concentrations of carbon dioxide (60—70 %). Furthermore, THORNTON (1933 b) showed that exposure to 50—70 % carbon dioxide for 1—5 days at 25° C. reduced the hydrogen ion concentration of the juice squeezed from the plant tissues. Storage of the tissues in air for some days after the treatment resulted in the restoration of the pH value to the same level as that of the control tissue. In his experiments on potatoes, the same author observed that, in the absence of oxygen, the pH change of the juices was not displaced to the alkaline side, but to that of increasing acidity.

HAMON (1936) studied the effect of 5—30 % carbon dioxide in the gaseous phase on the respiration of *Hypnum*, *Psalliota*, *Polyporus*, roses, violets, turnips, potatoes, and yeast. He found

that in certain plant tissues carbon dioxide caused an increased respiration, while in other tissues a slightly impeding effect was observed. In all cases, the effect was found to be reversible. In the experiments on yeast, filter paper covered with a thin layer of filtered and washed brewer's yeast was placed on the bottom of the experimental vessel. Under these conditions, the effect of carbon dioxide on the respiration of yeast was found to depend on the cultivation conditions of the yeast. Yeast from cultures grown at 4° C., from which carbon dioxide was not removed during cultivation, consumed less oxygen in the presence of carbon dioxide than in air. The opposite proved to be true when the yeast was cultivated at 24° C. and the carbon dioxide was removed from the nutrient solution. In comparison with yeast cultivated in air, the respiration of yeast cultivated under anaerobic conditions was only very slightly affected by 5—30 % carbon dioxide.

The effect of carbon dioxide on alcohol fermentation in yeast is rather insignificant. Only at a carbon dioxide pressure of some atmospheres can a slight checking of the fermentation be observed. LIESKE and HOFMANN (1929) have shown that fermentation at room temperature is not completely inhibited until a carbon dioxide pressure of 38—40 atmospheres (corresponding to a concentration of carbon dioxide in the nutrient solution of about 1 mol./l.). It may be mentioned here, on the other hand, that fermentation was found not to be affected by an oxygen or nitrogen pressure of 90 atmospheres. Even at a pressure of 1 000 atmospheres these gases did not bring about complete inhibition of the fermentation.

FIFE and FRAMPTON (1935) have observed that after treatment of sugar beet plants for one day in an atmosphere containing 20—60 % carbon dioxide, the pH of the hydraulically expelled juice of leaves, petioles and roots was essentially higher than in the corresponding parts of plants treated in air. After a short treatment, pure carbon dioxide caused an insignificant increase in pH; after longer treatment, however, the pH value became somewhat lower than the initial pH of the juice. Measurements on the juice from parts of the plants which had been allowed to recover in air for some time revealed that the observed changes in pH were largely reversible. It was found, furthermore, that the changes took place in the dark just as well as in light. Simultaneously with these pH changes, changes in the water-soluble nitrogen

fraction were observed; thus ammonia was liberated under the influence of carbon dioxide. FIFE and FRAMPTON also showed that the increase in pH as a response to carbon dioxide exposure appears to be a general type of response in plants, as ten other species of plants were found to behave in a similar manner. These authors supposed that the tissues could maintain their normal pH by means other than the buffering capacity of the cell sap, which was very low. FIFE and FERGUSON's (1941) studies revealed that the increase in ammonia obtained after treatment with carbon dioxide could be accounted for by changes in asparagine and glutamine amide-nitrogen.

D. Cytological Observations of the Effect of Carbon Dioxide.

It should be noted that numerous cytological investigations into various kinds of cells have also proved that moderately high carbon dioxide pressures reversibly affect the state and properties of the protoplasm. LOPRIORE (1895), in his experiments on *Tradescantia virginica*, found that the presence of 10—20 % carbon dioxide in the gaseous phase instantaneously checked, though not permanently, the streaming of the protoplasm in the anther filaments. The same author also investigated the effect of carbon dioxide on sporangium formation and the development of spores in *Mucor*, the reproduction of yeast, and the growth of pollinic tubes in a great number of plants. On the basis of these observations, LOPRIORE suggested, a view supported by some later workers, that living plant cells might counteract the disturbing effect of carbon dioxide, *i. e.* accommodate themselves to atmospheres with relatively high carbon dioxide content.

In experiments on animal cells the effect of carbon dioxide can also be directly observed. Some few examples from this field will be mentioned. CLOWES and SMITH (1922), SMITH and CLOWES (1924), and HAYWOOD and Root (1930) showed that slightly increased carbon dioxide pressure already checked the cleavage of eggs of *Arbacia* and *Asterias*. HAYWOOD (1927) treated newly fertilized *Arbacia* eggs in sea-water for a short time with 30—80 % carbon dioxide in the gaseous phase; the eggs were subsequently transferred to normal sea-water. After the egg had been treated only for 5 minutes, a markedly narcotic effect could already be detected; after this treatment, the time before the first cleavage

was prolonged. HAYWOOD (1925) showed, furthermore, that small concentrations of carbon dioxide impeded the movements of the branchial cilia of *Mytilus edulis*. CHASE and GLASER (1930) found that the effect of small concentrations of carbon dioxide on *Paramaecium* accelerated the movements of these cells (cf. ROOT's observations on the depressing effect of carbon dioxide on the respiration of the *Arbacia* egg, in contrast to its promoting effect on the respiration of *Paramaecium* at a carbon dioxide pressure of less than 50 mm. Hg).

JACOBS (1922) has studied the effect of carbon dioxide on the viscosity of the protoplasm. On centrifuging *Paramaecium*, *Colpidium*, the *Arbacia* egg, and *Spirogyra*, and studying the amoeboid movements in *Amoeba*, he found that treatment of the cells for a few minutes with carbon dioxide caused a decrease in viscosity. However, the viscosity again increased after continued exposure to carbon dioxide. Both effects were reversible, provided that the cells were not exposed to carbon dioxide for too long a time.

Numerous investigations have been made in which the effect of carbon dioxide on different experimental objects has been compared with that of small amounts of strong mineral acids or organic acids. In all cases, the effect of carbon dioxide at the same pH in the suspension medium has been found to be essentially greater and more reversible than that produced by addition of other acids. Such results have been obtained by, among others, JACOBS (1922), CLOWES and SMITH (1922), HAYWOOD (1925), and ROOT (1930) (cf. above), as well as by JACOBS (1920 a) who studied the toxic effect of different acids on toad tadpoles and protozoa. Further evidence was procured by the investigations made by FRASER (1925) on isolated strips of beating rabbit intestine, by SMITH (1926) on the contraction of turtle heart muscle, by BARTH (1929) on the coagulation and viscosity of the protoplasm in the *Arbacia* egg and, finally, by BECKER (1936), who studied the cessation of movements in the hairs of the anther filaments of *Tradescantia virginica* and *Paramaecium*. In this latter work may also be found reviews of a number of papers written in the Russian language, which support the view that the effect of carbon dioxide deviates essentially from that of other acids.

The cause of the comparatively vigorous effect of carbon dioxide on living cells has generally been ascribed to the ability of carbon dioxide to enter the cells much more rapidly than other acids, particularly the highly ionized mineral acids. Even compared to

oxygen, nitrogen and other gases, carbon dioxide permeates cells much more rapidly. This property may to some extent be due to the high solubility of carbon dioxide in lipids. More detailed data concerning the permeability of different cells to carbon dioxide, different acids and gases may be found in the papers by KROGH (1919), JACOBS (1920 b), SMITH (1926), BECKER (1936) and in the monograph of BROOKS and BROOKS (1941) on the permeability of living cells.

In interpreting the above observations concerning the effect of carbon dioxide on living cells, it is tempting to suppose that carbon dioxide causes changes in the acidity of the protoplasm and thus affects the viscosity and the metabolism. In order to elucidate this question, JACOBS, CHAMBERS, POLLACK, SPEK, BECKER and others have worked on cell material containing either natural indicators such as anthocyanines, or indicators supplied by vital staining. In this way, it has been possible approximately to follow the changes in pH after treatment of certain experimental objects with carbon dioxide or different acids which, in some experiments, were introduced into the cells by micro-injection. These experiments indicate that carbon dioxide, even if it enters the cell from a neutral or alkaline solution, frequently causes a decrease in pH. The effect of the gas, however, cannot exclusively be ascribed to the small changes in acidity of the protoplasm which were observed at moderate concentrations of carbon dioxide; it must also be due to a more or less direct effect on the protoplasm. (Further details on this subject may be found in BECKER.) In this connection, THORNTON's and FIFE and FRAMPTON's above-mentioned observations should be kept in mind. These authors pointed out that under the influence of carbon dioxide the pH of plant juice tends in certain cases to increase and not to decrease. This result agrees with LORPIORE's view that the cell is capable of counteracting a moderate effect of carbon dioxide; it complies, moreover, with the present author's observations on the effect of carbon dioxide on the pH in yeast cells communicated in this work (Chapter V).

It is at present unknown in what way carbon dioxide affects the state of the protoplasm and its function, except for its probable effect on the pH. From their observations concerning the reversible effect of carbon dioxide on the conductivity of electro-dialysed proteins, ADOLF and PAULI (1924) have gathered that a reversible compound is formed of both bicarbonate and hydrogen

ions with proteins. Even in discussions of the state in which carbon dioxide is present in the cell, it has been assumed by various investigators that such a combination might occur. Experiments on blood, for example, made it clear that carbon dioxide was not only found as the anhydrous form, as undissociated carbonic acid and as bicarbonate ions, but also bound to globin as a carbamate compound (cf. ROUGHTON 1935). Carbon dioxide may probably be bound to protein in yet another manner (SIDWELL, MUNCH, BARRON and HOGNESS 1938). This view has been strongly supported by discrepancies found in investigations of the behaviour of carbon dioxide in DONNAN equilibria and measurements of DONNAN potentials (ADAIR and ADAIR 1934, HENRIQUES 1931, and others) as well as by observations of carbon dioxide binding in the blood of certain fishes (WILLMER 1934 and FERGUSON, HORVATH and PAPPENHEIMER 1938). For further details see Chapter VI.

CHAPTER II.

General Methods and Materials.

A. Treatment of the Material and Methods of Analysis.

1. Experimental Details.

In the present investigations, baker's yeast (top yeast, *Saccharomyces cerevisiae*) has been employed as an experimental material. Before use, the yeast was washed by centrifuging from distilled water. Since the yeast, in suspensions at room temperature, soon exhibits clearly changed physiological features, the times of pretreatment, washing, preparation of the suspensions and their treatment were kept as constant as possible. After pretreatment of the yeast (see below), the suspensions were generally cooled in cold water, centrifuged in ice-cold tubes, and washed with cooled distilled water. The yeast was then immediately suspended in a succinate-succinic acid buffer.

In the calculations, the values of all analyses are given per gram of wet weight of original baker's yeast. Details of the change in the initial dry weight of baker's yeast obtained after its pretreatment and probable errors based on the said calculation method are given in Section C of this chapter.

In view of the varying character of the experiments, their performance was adapted to the methods used in the analyses. It was attempted to attain as comparable and satisfactory gas exchange in the suspensions as possible. In experiments under aerobic conditions, the suspensions were shaken in large culture flasks with plane bottoms (volume 1.8 litre). When the suspensions were treated under anaerobic conditions, similar experiments were performed in the WARBURG apparatus, in view of the difficulty in rapidly obtaining anaerobic conditions within the culture flasks. For this procedure, trough-shaped flasks with one side bulb, but without a central tube, were employed. A series of flasks was

filled with the same suspension, substrate and gas mixture. After different periods of identical treatment, the contents of the apparatuses were fixed and analysed. In order to provide double determinations, two flasks were taken at each time interval.

All experiments were performed with shaking of the culture flasks or the WARBURG apparatuses in the water thermostat at a temperature of $25.0^{\circ} \pm 0.1^{\circ}$ C.

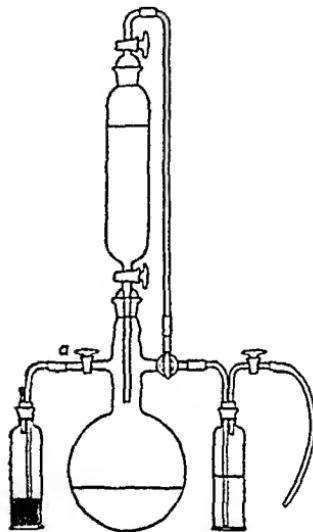


Fig. 2. Apparatus for preparation of CO_2 .

All saturations with gas were carried out at 25° C. while the suspensions were shaken in the thermostat. The gas mixtures of oxygen, nitrogen and carbon dioxide were prepared in cylindrical gasometers containing 1 litre (graduated in 10 ml., accuracy of readings 0.25 %), containing a saturated rock-salt solution. Both oxygen and carbon dioxide were taken from cylinders. The nitrogen used contained c. 1 % oxygen. In experiments under anaerobic conditions, and in those performed with gas mixtures of low oxygen pressure, nitrogen was used which had been freed from oxygen by leading over copper gauze spirals at 400° C. and washing with distilled water.

For the determinations of the ability to bind carbon dioxide in the yeast, carbon dioxide was prepared by adding a saturated solution of chemically pure potash dropwise to chemically pure 50 % sulphuric acid. The apparatus used for these experiments is illustrated in Fig. 2.

After the flask and separating funnel were filled, they were treated through tube "a" for c. 2 hours with carbon dioxide from a cylinder. Some litres of carbon dioxide were developed in the apparatus before its use as a carbon dioxide source. The carbon dioxide obtained was regarded as chemically pure, containing c. 100 vol. % carbon dioxide. Test experiments carried out with carbon dioxide from the apparatus described and from a cylinder led to the same result regarding the effect of carbon dioxide on yeast. In experiments concerning the effect of carbon dioxide on the metabolism of yeast and in some of the determinations of the amount of carbon dioxide bound within the cells, the more convenient and cheaper cylinder carbon dioxide was therefore em-

ployed. In view of the considerable solubility of carbon dioxide even in saturated rock-salt solution, the solution within the gasometers was saturated with each gas mixture by shaking at the pressure of the gas components in question. This pretreatment was always performed 3 to 4 times before the gasometers were finally filled.

In all experiments with WARBURG apparatuses, the additions were made with KROGH syringes calibrated at room temperature; the pipetting errors were less than 0.5 %.

Besides KROGH syringes, calibrated pipettes were used for taking the samples and making dilutions for the analyses.

2. Succinate-Succinic Acid Buffer (SS Buffer).

Following a suggestion by SPERBER, we have used for some years a sodium succinate-succinic acid buffer at pH 5 as a suspension medium in investigations of the metabolism of yeast (see RUNNSTRÖM and SPERBER 1938 a). In numerous experiments with succinate buffer of the said acidity, this was obviously not attacked by the baker's yeast used, nor did it prove to poison the yeast (cf. RUNNSTRÖM, BOREI and SPERBER 1939).

For the experiments subsequently described, more detailed data concerning the buffer were desirable. The pH values of different mixtures of succinic acid and sodium hydroxide, and the changes in the pH during dilution of the buffer, were measured. Furthermore, the changes in the pH during saturation of the buffer with gas mixtures of various carbon dioxide contents were examined. Following the practice in previous publications, the succinate-succinic acid buffer will henceforth be denoted as *SS buffer*.

As stock solutions, buffers were prepared which were *M/5* with respect to succinic acid (*pro analysi*, SCHERING-KAHLBAUM). Sodium hydroxide was made from concentrated lye diluted with CO₂-free distilled water. The pH was determined with a glass electrode, using as reference solution a standard solution of phosphate buffer, pH 6.50, and a VEIBEL solution, pH 2.04. The apparatus applied was a valve potentiometer (RADIOMETER, Copenhagen).

Table 1 contains the pH values measured at 19—20° C. The buffer was prepared from 11.80 g. succinic acid (= 0.1 mol.) + sodium hydroxide; the mixture was then diluted with distilled water to 500, 1 000 and 1 500 ml. In this manner, *M/5*, *M/10* and *M/15* Na-SS buffers were obtained. A pH indicates the difference between the pH values of *M/5* and *M/15* buffer.

Table 1.
Na-SS buffer. pH value measured at 19-20° C.

Succinic acid	Addition of	pH			ΔpH $M/5 - M/15$
		M/5	M/10	M/15	
11.8 g.	70.0 ml. 2.668 N NaOH	5.98	6.05	6.08	0.10
11.8 g.	60.0 " 2.668 N "	5.40	5.45	5.49	0.09
11.8 g.	50.0 " 2.668 N "	5.05	5.11	5.14	0.09
11.8 g.	35.0 " 2.668 N "	4.52	4.59	4.61	0.09
11.8 g.	20.0 " 2.668 N "	4.02	4.07	4.08	0.06
11.8 g.	8.0 " 2.668 N "	3.49	3.51	3.54	0.05
11.8 g.	0.0 " 2.668 N "	2.45	2.57	2.66	0.21

Fig. 3 represents the curve for $M/15$ Na-SS buffer. While the difference between the first and the second dissociation constants for succinic acid is rather small, the transition from the first to the second state of protolysis shows no inflection in the curve. This result was confirmed by repeated electrometric titrations of succinic acid with sodium hydroxide.

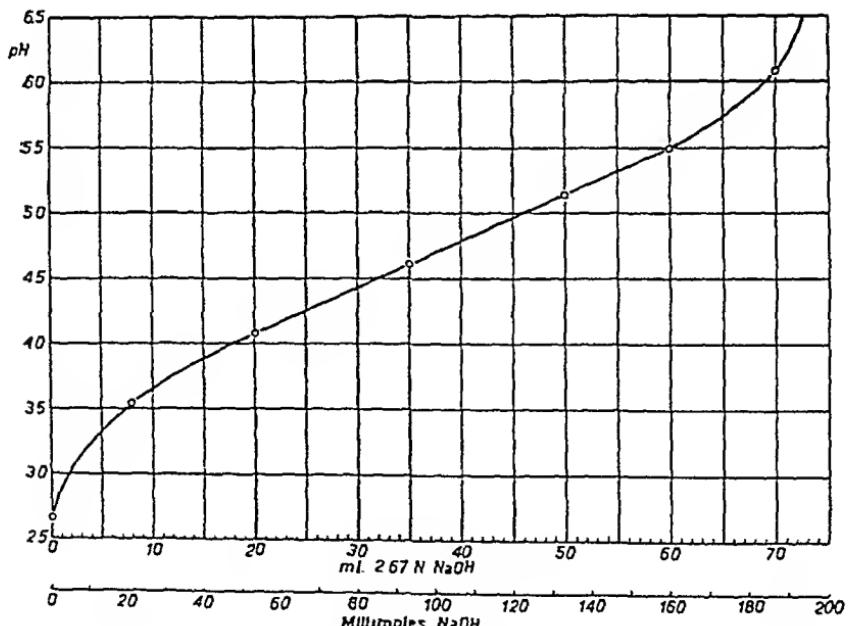


Fig. 3. pH of $M/15$ Na-SS buffer prepared from 11.80 g. (= 0.1 mol.) succinic acid + 2.67 N NaOH + H_2O to 1500 ml.

Fig. 4 illustrates the effect of dilution on pH of the Na-SS buffer at pH 5.1.

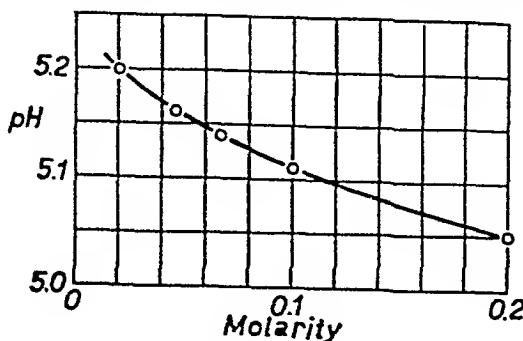


Fig. 4. Effect of dilution on pH of Na-SS buffer.

In the study of the power of yeast for binding carbon dioxide, the effects of potassium and calcium ions were also examined. In the majority of experiments, a buffer at about pH 5.1 was used. The sodium ions in this buffer were exchanged for equivalent amounts of potassium and calcium ions. The pH values of these SS buffers diverged to some extent from those in the corresponding Na-SS buffer. On changing the amount of succinic acid added, approximately the same values were obtained for the pH of the potassium and calcium buffers and the corresponding sodium buffer. This is illustrated in Table 2. On account of the variation in the amount of succinic acid, the concentrations given at the head of the table are only approximate. The measurements were performed at c. 18° C.

Table 2.

pH values after exchange of Na ions with K and Ca ions in the buffers.

Succinic acid	Addition of	pH		
		M/5	M/10	M/15
11.80 g.	50 ml. 2.668 N NaOH (0.133 equiv.)	5.05	5.11	5.14
11.80 g.	50 ml. 2.672 N KOH (0.133)	5.12	5.14	5.17
11.80 g.	50 ml. 2.700 N KOH (0.135)	—	5.17	5.20
12.20 g.	50 ml. 2.700 N KOH (0.135)	—	5.09	5.13
11.80 g.	6.70 g. CaCO ₃ (puriss.) (0.134)	— ¹	4.88	4.93
10.80 g.	6.70 g. CaCO ₃ (puriss.) (0.134)	— ¹	5.02	5.08

¹ Ca succinate not sufficiently soluble.

In the investigations of the metabolic effect of carbon dioxide on yeast and in determinations of the capacity of yeast for binding carbon dioxide, the suspensions of yeast in the buffer were treated

at atmospheric pressure with gas mixtures containing up to 50 % carbon dioxide. According to experiments carried out by MALM (unpublished) at this Institute, a slight change of the pH in acid direction was obtained in the suspension medium when baker's yeast was suspended in non-buffered salt solutions. As considerable amounts of yeast were necessary for the manometric determination of bound carbon dioxide, the pH of the buffer was tested in the presence of yeast and in an atmosphere containing carbon dioxide. The buffer concentrations and the amounts of suspended yeast used in these experiments correspond approximately to those in the determinations of carbon dioxide bound in yeast. (No corrections were made, however, for the change of buffer concentration due to the dilution of the buffer with the intercellular water of the yeast.) In Table 3 are collected the pH values measured in yeast suspensions and in the buffer at equilibrium with a mixture of carbon dioxide and air. The temperature during the measurements was 20° C. Δ pH denotes the difference between the pH measured in air and that in the presence of 50 % carbon dioxide in the gaseous phase.

Table 3.

pH in yeast suspensions and in Na-SS buffer at equilibrium with mixtures of CO₂ and air.

M/10 Na-SS buffer		Distilled water	Washed baker's yeast	pH measured when the gaseous phase contained			Δ pH air—50 % CO ₂
ml.	pH			ml.	g. wet weight	air	
20	5.11	10	—	5.14	—	5.12	— 0.02
16	5.11	10	4	5.14	—	5.12	— 0.02
20	5.45	10	—	5.49	5.47	5.46	— 0.03
16	5.45	10	4	5.43	—	5.41	— 0.02
20	6.05	10	—	6.08	5.94	5.84	— 0.24
16	6.05	10	4	6.02	—	5.77	— 0.25

From Table 3 it is clear that the pH decreases by about 0.06 units when 4 g. of yeast is suspended in 16 ml. of the two latter buffer mixtures. In all suspensions, the concentration of the buffer becomes 0.059 M, provided that the intercellular water content of the yeast after washing is 32 % of its wet weight (cf. Section C of the present chapter). In the first case, this dilution should

cause an increase in the pH of c. 0.01 pH units, which can be neglected. When the buffer at pH 6 and the yeast suspensions in this buffer are treated with a gas mixture containing 50 % carbon dioxide, the pH decreases by $\frac{1}{4}$ pH unit owing to the formation of bicarbonate. In the buffer solutions with lower pH, the pH values are decreased only insignificantly when the solutions are brought into equilibrium with the gas mixtures containing carbon dioxide.

3. Methods of Analysis.

Manometric methods. Determinations of the metabolism of yeast under different physiological conditions and of the amounts of carbon dioxide bound in the yeast were performed by the manometric method of WARBURG, modified for the purpose in hand. The metabolism of different yeasts under aerobic and anaerobic conditions was studied in cases where carbon dioxide was not added to the gaseous phase, according to the so-called direct method. Conical flasks with a central tube and two side bulbs were used in these determinations. The yeast suspension in Na-SS buffer at pH 5.1 was pipetted into the main compartment of the vessel, the substrate into the side bulbs. After tilting, the volume of the liquid in the main compartment amounted to 2.40 ml., the final concentration of the SS buffer being c. 0.08 M. At pH 5.1, oxygen consumption and carbon dioxide production could be measured in a pair of equal flasks without paying regard to the carbon dioxide retention. The oxygen consumption was measured directly in the flask where the carbon dioxide evolved was absorbed by 0.3 ml. 10 % potassium hydroxide in the central tube. In order to increase the absorbing surface, a piece of folded filter paper was placed in the potassium hydroxide, protruding above the liquid surface. In the corresponding pair of apparatuses, the central tube contained 0.30 ml. distilled water, and the changes in pressure due to oxygen consumption and carbon dioxide production were measured. If the pressure change due to oxygen consumption was known from measurements in the preceding apparatus, the amount of carbon dioxide produced could be calculated. The amounts of oxygen and carbon dioxide were calculated as usual for 0° and 760 mm. Hg.

For the determination of the endogenous metabolism of yeast, in an atmosphere containing carbon dioxide, WARBURG's indirect

method was applied. This method is based upon the fact that when two flasks containing different liquid volumes are used, different pressures are obtained owing to the different solubilities of oxygen and carbon dioxide in the suspension medium. The experiments were carried out in trough-shaped vessels with one side bulb, but without a central tube. The suspensions in the two flasks contained 100 mg. of washed baker's yeast in 1.0 ml. *M/5* and 4.0 ml. *M/20* Na-SS buffer, pH 5.1, respectively. The rate of shaking was 120 complete oscillations per minute. In view of the limited accuracy of the method, each experiment was performed in duplicate with two pairs of determinations in each atmosphere, with and without carbon dioxide, and the mean value for each pair was calculated. Two flasks, one containing 1.0 and the other 4.0 ml. *M/10* buffer, which were filled with an atmosphere corresponding to that in the apparatuses with yeast, served as thermo-barometers. The flasks were treated, with shaking, for about 25 minutes in the thermostat with 3 litres of a carbon dioxide mixture for each coupled series of six flasks. The first reading was made 10—15 minutes after the treatment with gas was terminated. Control experiments were performed in air. When the measurements were begun, the yeast had thus been shaken in air or in an atmosphere containing carbon dioxide for about 40 minutes. In the calculation of oxygen consumed and carbon dioxide developed, the following values for the absorption coefficients of the gases were employed: $\alpha_{O_2}^{25^\circ} = 0.028$ and $\alpha_{CO_2}^{25^\circ} = 0.757$, which are the values for water. In checking the calculations of the experimental results, other values were used (cf. Chapter IV), *viz.* for *M/5* buffer $\alpha_{CO_2}^{25^\circ} = 0.747$, and for *M/20* buffer $\alpha_{CO_2}^{25^\circ} = 0.794$. The oxygen and carbon dioxide values calculated by means of the later α_{CO_2} values, however, do not deviate essentially from the earlier ones. For a more detailed description of the manometric methods and the calculations, see DICKENS (1940) and DIXON (1943).

The manometric determination of the amount of acid-labile bound carbon dioxide in yeast is described in Chapter IV in relation to experiments aimed at the improvement of this method.

Phosphate. When phosphate was determined in yeast, the suspensions were fixed with trichloroacetic acid in such a way as to give a final concentration of 4 %. The total amount of phosphate was obtained after burning an aliquot part of the sus-

pension in special KJELDAHL flasks with long necks of 25 ml. volume. The combustions were performed with 0.30 ml. concentrated sulphuric acid (*p. a.*) after addition of perhydrol (MERCK). The heating was carried out in an electric stove. After complete combustion, the contents of each flask were diluted with 5 ml. distilled water and boiled in order to destroy any pyrophosphate formed.

The suspensions fixed with trichloroacetic acid were centrifuged after 45 minutes at room temperature. "*Free acid-soluble phosphate*" was determined in the clear liquid. The total amount of phosphate in the extract was burnt, as described above. The difference between the value thus estimated and that for free phosphate is denoted as "*bound acid-soluble phosphate*". Hydrolysis experiments were performed in normal sulphuric acid at 100° C. The phosphate estimations were performed in double samples by BRIGG's (1924) colorimetric method. The extinction was measured in the PULFRICH step-photometer (red filter S 72) after exactly 60 minutes. (For details concerning the development of the colour as a function of time, see BOREI 1943.) The analyses are corrected in respect of probable absorption by means of blanks containing buffer, trichloroacetic acid and sulphuric acid, and are always expressed in terms of phosphorus.

Nitrogen. Determinations of the total content of nitrogen in yeast and the amount of "*acid-soluble nitrogen*" were made after combustion of suspensions fixed with trichloroacetic acid and of centrifuged extract, respectively, obtained in the same way as described for the phosphorus estimations. The combustions were performed after addition of 0.60 ml. concentrated sulphuric acid. Instead of perhydrol, potassium persulphate was used (N-free; *p. a.*); which proved to be better suited to the combustions in the presence of the relatively small amounts of sulphuric acid used. The amount of "*ammonia nitrogen*" in yeast was determined by distilling both the fixed suspension and the centrifuged extract. "*Amide-nitrogen*" was found by distillation after hydrolysis of fixed suspensions in normal sulphuric acid for 4 hours at 100° C. In the determinations of the uptake of ammonia nitrogen by yeast during the turnover of glucose in the presence of ammonium chloride, the metabolic processes in the suspensions were interrupted by rapid cooling and centrifuging in ice-cold tubes, whereupon the remaining ammonia was determined in the suspension medium.

All distillations were performed *in vacuo*, applying the modified PARNAS-WAGNER apparatus with ground joints according to TEORELL (1932) (cf. PARNAS and HELLER 1924). The samples from the combustions were made alkaline by means of sodium hydroxide in the distillation apparatus. Distillations of fixed suspensions, extracts, hydrolysates or suspension media for the determination of ammonia were carried out at pH 9.2 after neutralization with hydroxide and addition of borate buffer (PARNAS and HELLER 1924). In all cases, the receiver contained hydrochloric acid. Total nitrogen was determined by titration of the hydrochloric acid with CO₂-free N/100 sodium hydroxide and using the TASHIRO indicator according to CONWAY and BYRNE (1933). Acid-soluble nitrogen and ammonia nitrogen were determined by nesslerization, *i. e.* 12—15 ml. were distilled over and the distillate was made up to 20 ml. with permutite-treated distilled water. 2 ml. of NESSLER's reagent was then added (see PETERS and VAN SLYKE 1932). The extinction was determined in a LANGE photoelectric colorimeter with a blue filter. Just as in the phosphate estimations, a standard curve was always constructed simultaneously with each series of estimations. The values obtained in the nitrogen determinations generally represent the mean values of analyses performed on three parallel samples measured against a distilled blank.

Alcohol. Estimations of alcohol produced during fermentation were performed according to FRIEDEMANN and KLAAS (1936).

Glucose. In the determinations of the consumption of glucose in suspensions of yeast metabolizing glucose in the presence and absence of ammonium chloride, the uptake of glucose was interrupted at different intervals. A 1.0 ml. sample was taken from the suspension by means of a KROGH syringe and directly ejected into a centrifuge tube containing 10.0 ml. ice-cold distilled water. After shaking, the mixture was centrifuged immediately. Part of the clear liquid was pipetted off and was allowed to attain room temperature. The glucose was then determined in aliquot parts of the solution. When not otherwise stated, glucose estimations were performed by the colorimetric semi-micro method of BENEDICT (1931). The extinction was measured in the step-photometer (red filter S 72). In certain other experiments, glucose was determined by the method of WILLSTÄTTER and SCHUDEL (1918), modified according to GOEBEL (1927).

Pyruvic acid. The uptake of pyruvic acid by yeast was also determined, as described above, by analysing the suspension medium after centrifuging off the yeast. 5.0 ml. suspension was ejected into 5.0 ml. ice-cold water and centrifuged immediately. The clear liquid was analysed for pyruvic acid by addition of ceric sulphate solution and back-titration with N/100 ferrous sulphate according to the method of FROMAGEOT and DESNUELLE (1935). Erioglaucin A served as indicator (see KOLTHOFF and MENZEL 1931 and RUNNSTRÖM and SPERBER 1941). Before preparing the solutions of the pyruvic acid (MERCK) used, the acid was redistilled several times *in vacuo*.

Growth. In growth determinations, yeast was cultivated in a nutritive solution of non-hopped wort + WILLIAMS (1920) solution containing 1 % glucose in a 1 : 8 mixture. By this procedure, a nutritive solution was obtained containing c. 2 % sugar. Previous to mixing with WILLIAMS solution, the wort was autoclaved and filtered through a SEITZ filter. The nutritive solution was subsequently also autoclaved. The experiments were performed in 450 ml. culture flasks (FERNBACH flasks) under shaking at 25° C. The composition of the modified WILLIAMS solution is seen in Table 4.

Table 4.

Composition of modified WILLIAMS solution containing 1 % glucose.

KH ₂ PO ₄	2.0	g.
CaCl ₂	0.25	"
MgSO ₄ · 7 H ₂ O	0.50	"
(NH ₄) ₂ SO ₄	3.0	"
Glucose	10.0	"
Asparagine	1.5	"

Dissolved in distilled water to 1 000 ml.

The dry weight of the yeast before and after incubation was taken as a measure of the extent of growth. After the yeast had been sucked off and washed on Jena sintered glass crucibles 1 G 4, its dry weight was determined after drying at 105° C. for 20 hours.

B. Yeasts Employed.

1. Manufactured Yeasts.

In the present investigations, baker's yeast has been employed as the primary material. It was received immediately after manufacture from the Swedish Yeast Factory Co. at Rotebro, Stockholm (Svenska Jästfabriks A.B.).

The manufacture of baker's yeast was carried out by the method known as differential fermentation. In this procedure, the yeast was cultivated on beet molasses and nutritive salts. The cultivation was performed in a series of consecutive cultures denoted by Z 1—Z 5. From the ready Z 5 cultivation, baker's yeast was separated by sucking off on rotating filter drums. The so-called mother yeasts obtained from different cultures are denoted by Z 1—Z 4. Because of the treatment during cultivation the three first mother yeasts (Z 1—Z 3) assumed a more or less anaerobic character. If not otherwise mentioned, the experiments were carried out with the same type of yeast, corresponding to type B in the paper of BOREI and SJÖDÉN (1943). In agreement with these authors' nomenclature, a type cultivated earlier is henceforth denoted by A, which corresponds to the denotation D formerly used by RUNNSTRÖM and co-workers.

Baker's yeast (Z 5). This yeast was cultivated during very vigorous aeration. Dry weight 25—27 %. Protein content 46—47 % of the dry weight. This type is practically free of other organisms, such as other fungi and bacteria. At the end of the cultivation, the nutritive solution contains a small excess of carbohydrate relative to the amount of nitrogen source added. The growth was therefore terminated and the yeast contained but a few buds. In contrast to growing yeast, this completed baker's yeast can be denoted as a ripe or "stationary" yeast. During the turnover of glucose by yeast, only weak aerobic fermentation but high respiration are obtained. The latter already reaches its maximum at low concentrations of glucose.

Z 1 yeast was manufactured by cultivating the yeast under anaerobic conditions and adding molasses and salts simultaneously and in relatively high concentrations. Under these circumstances, the growth processes in the yeast occurred parallel with the fermentation processes, causing a rapid formation of alcohol. The protein content amounted to c. 50 % of the dry weight of the yeast. In contrast to baker's yeast, the present yeast, under aerobic conditions, consumes glucose with vigorous fermentation and low respiration (see RUNNSTRÖM and SPERBER 1941).

Z 2 yeast is the denotation for the type obtained from the following cultivation. Just as the preceding yeast, it was not cultivated under continual addition of nutritive solution, but under weak aeration. The production of alcohol during propagation was

high. Approximately 60 % of the dry weight (22—23 %) of the filtered yeast consisted of protein. This yeast showed very intense budding.

Z 3 yeast is an unripe yeast of about the same protein content as the preceding type. During cultivation, the yeast was aerated rather weakly, and a relatively high alcohol formation consequently also occurred during the propagation of this yeast.

Z 4 yeast is the mother yeast preceding the final production of baker's yeast. The budding of this type was not completely terminated. The yeast was aerated vigorously and, as in the final cultivation of baker's yeast, no alcohol was formed during cultivation. The protein content was c. 50 % of the dry weight.

For information regarding the contents of reserve carbohydrates in the different mother yeasts, the reader is referred to BRANDT (1941 a) and to Section C of the present chapter.

2. Pretreated Baker's Yeasts.

By pretreating baker's yeast as described below, it was intended to obtain yeasts changed in a characteristic manner with regard to some physiological features. The changes occurring in baker's yeast during pretreatment and the qualities of pretreated yeasts are described in greater detail in Section C of this chapter.

Starred Yeast. 4 g. baker's yeast + 120 ml. $M/15$ Na-SS buffer, pH 5.1, were shaken under aerobic conditions in large culture flasks at 25° C. for 20—22 hours. The yeast was subsequently washed by centrifuging twice with distilled water.

Fed Yeast. 4 g. baker's yeast + 96 ml. $M/5$ Na-SS buffer, pH 5.1, + 80 ml. distilled water + 60 ml. 0.14 M glucose solution (= 1.5 g. glucose) were shaken under aerobic conditions in large culture flasks at 25° C. for $2\frac{1}{2}$ hours. The suspension (240 ml.) was cooled, centrifuged immediately in ice-cold centrifuge tubes and washed by centrifuging three times from cold water.

Budding Yeast. 4 g. baker's yeast + 96 ml. $M/5$ Na-SS buffer, pH 5.1, + 20 ml. distilled water + 60 ml. 0.14 M glucose solution + 60 ml. 0.042 M ammonium chloride solution (= 135 mg. NH_4Cl) were treated in the manner described for the preceding yeast.

When calculating the initial concentrations in the mixtures used in the pretreatment of baker's yeast for the preparation of

fed and budding yeasts, we obtain 1.67 % baker's yeast (calculated from the wet weight), 0.0347 *M* glucose, 0.0105 *M* ammonium chloride, and 0.080 *M* Na-SS buffer.

The preparation of yeasts pretreated with glucose and cysteine or in the presence of certain cell poisons is described below, together with the experiments on these yeasts.

Dried Yeast. This yeast was prepared from washed baker's yeast which was pressed in a hand-operated screw press in order to remove as much water as possible. The yeast was subsequently pressed through a fine-meshed sieve and spread on filter paper or stainless steel plates in a very thin layer. It was then dried at room temperature in the draught cupboard. The dried yeast contained c. 8 % water. The yeast was stored in the refrigerator. In the calculations, 280 mg. dried yeast is considered to correspond to 1.0 g. fresh baker's yeast.

C. Effect of Pretreatment on Yeast. Characteristics of Pretreated Yeasts.

The pretreatments of baker's yeast described in the preceding chapter cause characteristic alterations in the cells and their physiological properties. For an examination of the observations described and discussed in the following chapters, it was of great value to know the changes, originating from different pretreatments, in certain plasma components, and the properties of the protoplasm probably conditioned thereby, such as alterations in the endogenous respiration and the fermentation. Only those substances are considered which occur quantitatively in such concentrations that they can be assumed to be of immediate importance as substrates in the endogenous metabolism or to influence the binding of carbon dioxide and the acidity of the protoplasm. These experiments aim at the elucidation of changes in the physiological state of the cells obtained at the transition from the so-called "stationary state" of baker's yeast to aerobic carbohydrate metabolism or to an incipient growth, such as is manifested by the assimilation of ammonia nitrogen in the presence of glucose. Cytological changes were investigated in which the appearance of nucleotides in the cytoplasm could be followed. Furthermore, the uptake of both glucose and ammonia and the resulting changes in the content of reserve carbohydrates, which

latter cause some changes in the endogenous metabolism of the cells, were studied. Finally, the changes in certain nitrogen-containing fractions and the effect of pretreatment on the distribution of phosphate compounds in the protoplasm were determined.

The experiments, on which the data given below are based, were performed at different times in the course of several years, during which period a certain variation in the physiological properties of the yeast was unavoidable. Comparatively small variations in the yeast could be observed from one day to another. In the investigations that were extended over longer periods or repeated after several months or a year, however, discrepancies were observed in some cases, caused by variations in the manufacture and changes in the raw material as, for example, differences in the quality of the molasses, in the length of its storage period, and in the details of the technical procedure. The absolute values of the rate of the metabolism or the concentration of various constituents of the protoplasm are therefore not always quite comparable. The behaviour, deviating from that of baker's yeast, observed during or after different pretreatments of this material were, however, always investigated in parallel to that of the initial yeast. The absolute values given for the concentrations of certain constituents of the protoplasm should only be considered an approximate measure of the proportions of these constituents and of the changes caused by the pretreatment. The essential point was to show the occurrence and the nature of the alterations caused by pretreatment.

1. Cytological Examination.

In investigations of the different types of yeast, the appearance of the living cells in visible light was continuously checked under the microscope. The appearance of the protoplasm provides a suitable basis for checking the normal reaction of the cells to different modes of pretreatment. The knowledge on which the conclusions were based was mainly gained by experiments on the living cell in ultraviolet light. In collaboration with CASPERSSON, the part played by nucleic acids in the growth of yeast was primarily studied (CASPERSSON and BRANDT 1941).

A brief review of certain cytological alterations during different pretreatments will now be given. Micro-photographs of living yeast cells in culture media were taken with an apparatus constructed

by CASPERSSON after the designs of KÖHLER (1904) and KÖHLER and TOBGY (1928). An objective (monochromat 1.66 mm.) specially corrected for the wave-length 257 m μ . (cadmium electrodes) was employed. The numerical aperture at this wave-length is 1.25, corresponding to a resolving power more than twice that which can be obtained with visible light. For more detailed information of the investigations in ultraviolet light, the cytology of baker's yeast, and the discussion of the relevant cytological problems, the papers of CASPERSSON and BRANDT (1941) and BRANDT (1941 b) should be consulted.

Fig. 5 shows a photograph of living cells of ripe baker's yeast taken at 257 m μ . The cells, being ellipsoidal or spherical, are of rather varying size. The maximum length of the cells is normally 8—10 μ . In baker's yeast only very few budding cells can be detected. The hyaloplasm is almost homogeneous and shows a relatively weak absorption. The vacuoles appear clearly, especially in bigger cells in which a single large vacuole is usually found. Strongly absorbing granules of somewhat irregular form are clearly visible; in the hyaloplasm they appear sometimes spread, sometimes "aggregated", and usually adjacent and surrounding the vacuole. They are never observed within the vacuoles. The granules correspond to MEYER's (1904) so-called volutine grains. In the literature they are also given the less suitable denotation "metachromatic corpuscles" (see BRANDT 1941 b).

In agreement with MEYER's conception that volutine is a nucleic acid compound, CASPERSSON and BRANDT were able to show by absorption measurements in ultraviolet light that these grains contained considerable amounts of ribo-nucleotides. As the absorption maximum (260 m μ) of these compounds lies almost at the wave-length of the light employed here, and since the specific extinction coefficient of the nucleotides is very high, the changes in the distribution of the nucleotides within the cells appear very distinctly at 257 m μ . The volutine grains correspond to the granules within the hyaloplasm visible in ordinary light. These granules appear clearly because of their great refraction. Since no visible fat globules are present in the intact protoplasm, there is no risk of confusing volutine grains with these in visible light. Consequently, certain observations regarding nucleic acids in the ultraviolet can be made in the study of living cells in ordinary light.

Starvation of the yeast in the manner described above is followed by vacuolization of the cytoplasm. As seen in Fig. 6, nu-

Fig. 5. Baker's yeast. Living. The ultraviolet-absorbing volutine grains are partly aggregated and frequently situated adjacent to the vacuolar border. 1950 x. Wave-length 257 m μ . Objective: monochromat 1.66 mm. corrected for 257 m μ .



Fig. 6. Starved yeast. Living. The volutine grains clearly heaped together. The protoplasm rather vacuolized. 1950 x. Photo as in Fig. 5.

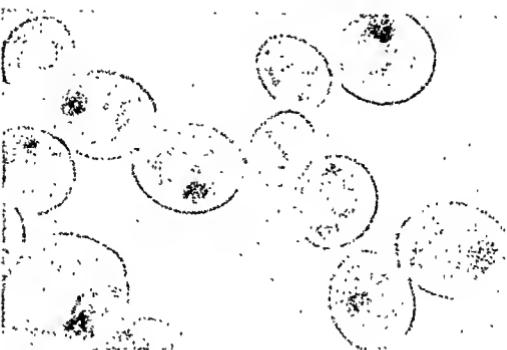


Fig. 7. Baker's yeast shaken under aerobic conditions for 2 $\frac{1}{2}$ hours in initially 0.2 % glucose solution at 25° C. Living. Volutine grains spread over the protoplasm. Vacuoles scarcely visible. 1950 x. Photo as in Fig. 5.



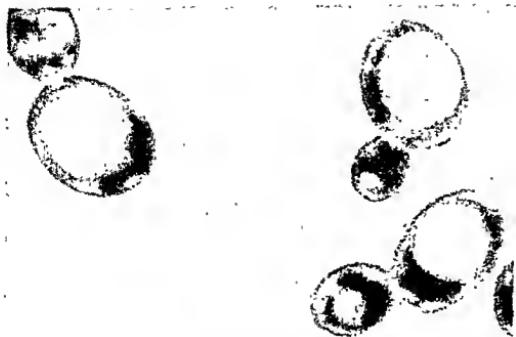


Fig. 8. Baker's yeast cultivated for $3\frac{1}{2}$ hours in nutrient solution containing wort at 25° C . Living. The volutine grains partly dissolved. The nucleotides have begun to spread over the strongly absorbing hyaloplasm both in the mother cell and the buds formed. Well developed vacuoles. $1950 \times$. Photo as in Fig. 5.

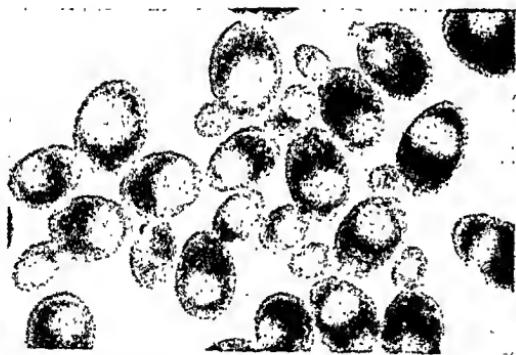


Fig. 9. Vigorously growing yeast cells from pure culture in nutrient solution containing wort. Living. Volutine grains dissolved and regularly distributed over the strongly absorbing hyaloplasm. Well developed vacuoles. $1220 \times$. Photo as in Fig. 5.

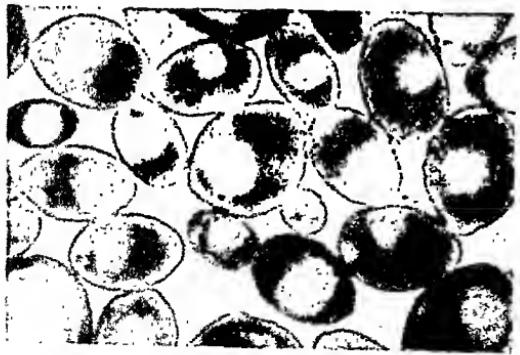


Fig. 10. Mother yeast Z 3 obtained in the propagation of commercial baker's yeast. Living. Volutine grains not quite dissolved. Hyaloplasm strongly absorbing. $1950 \times$. Photo as in Fig. 5.

merous vacuoles are clearly seen as small bright bubbles. The position of the volutine grains in starved yeast has also changed characteristically. In almost all cells, the volutine grains are associated to a compact and strongly absorbing group distinctly contrasting with the background of the weakly absorbing hyaloplasm.

The fed yeast obtained after consumption of glucose under aerobic conditions exhibits an opposite behaviour. The vacuoles are practically invisible in the cells and the volutine grains are regularly distributed within the hyaloplasm (Fig. 7).

After consuming glucose in the presence of ammonium chloride, the cells have quite a different aspect. Apart from the budding, which already begins after some hours, a progressive dissolution of the volutine grains is observed; simultaneously, the vacuoles become well developed. The interface between the grains and the hyaloplasm becomes indistinct and the absorption of the hyaloplasm increases. Fig. 8 shows an example of this, *viz.* baker's yeast cultivated at 25° C. for 3½ hours in WILLIAMS' solution + 10 % wort. During continuous cultivation of the cells an increase is obtained by repeated budding. When the grains are completely dissolved the hyaloplasm becomes rather homogeneous and strongly absorbing. This is illustrated by Fig. 9 which represents cells from a pure culture of baker's yeast. The vacuoles, which appear clearly as bright spheres within the cells, absorb only little light, thus indicating that the vacuolar fluid contains very little nucleotides. The appearance of the so-called budding yeast corresponds rather well to the yeast represented in Fig. 8 or in Fig. 10. The volutine grains are still clearly visible, and the vacuoles are generally somewhat smaller than is indicated in the figures.

During the investigation in ordinary light of the mother yeasts (denoted above as Z 1—Z 4 yeasts) received directly from the factory, it was observed that, despite the considerable proliferation of the yeast, the protoplasm contained small, extended, not quite dissolved volutine grains. In ultraviolet light, however, the hyaloplasm absorbs strongly, thus indicating that even within the protoplasm of these types of yeast relatively homogeneously distributed nucleotides are abundantly present. Fig. 10 shows the appearance of a Z 3 yeast.

CASPERSSON's measurements of the ultraviolet absorption spectrum of the protoplasm of baker's yeast and cells of pure

cultures of rapidly growing yeast (CASPERSSON and BRANDT 1941) showed that, as compared with the amount of protein (estimated as tryptophan and tyrosine), growing yeast includes remarkably more nucleotides than does baker's yeast with a stationary metabolism. Moreover, the amount of nucleic acid in the growing cells was observed to be considerably greater than in baker's yeast. The order of magnitude of the nucleotide content in the yeast was estimated to be about 1 % of its wet weight. The main part of this is ribo-nucleic acids (yeast nucleic acid). Apart from the above discussed dissolution of volutine grains during growth, a considerable fresh formation of nucleotides thus takes place, which presumably is intimately related to the synthetic processes causing the formation of protein during growth.

The phenomena associated with the ribo-nucleotides of yeast stand in concordance with observations, made primarily by CASPERSSON and his school, regarding the occurrence of heterochromatic regions in higher cells of animal origin (see, for example, CASPERSSON's review 1941). According to these studies, the volutine grains in the yeast can be regarded as equivalent to heterochromatin, while the small FEULGEN-positive elements present in the yeast may be considered the gene-bearing euchromatic chromosome equivalents. During the telophase in more highly organized cells, protein of the histone type, of which the nucleolus is constituted, occurs in connection with the heterochromatin. It is possible that an analogous formation of histone-like protein in yeast takes place in connection with the volutine grains during growth. This view is supported by the ultraviolet absorption curves for baker's yeast and growing yeast. The position of the absorption band indicates that the protein, presumably of the histone type, probably occurs in a higher concentration in or around the volutine grains than in the remaining protoplasm (see CASPERSSON and BRANDT 1941).

[2. Carbohydrate Metabolism.]

In the turnover of glucose by yeast under the conditions described for the preparation of fed and budding yeasts, part of the sugar is stored in the cell as reserve carbohydrates. In baker's yeast, these are present mainly as glycogen and trehalose. It is difficult analytically to determine the occurrence and the changes of the glycogen content in yeast. The yeast cells contain con-

siderable amounts of polysaccharides typical of the yeast, which are of a relatively unknown character and which appreciably disturb the glycogen analysis. Thus, the yeast contains an alkali-resistant mannan (HAWORTH, HIRST and ISHERWOOD 1937) precipitable with copper, which forms a main constituent of the so-called yeast gum (cf. HAWORTH, HEATH and PEAT 1941). From the untreated yeast, ZECHMEISTER and TÓTH (1934, 1936) extracted by means of alkali a glucan. This glucan contains the unusual 1,3-glucosidic linkage between the glucopyranose units in the chain (FREUDENBERG and PLANKENHORN 1938, and HASSID, JOSLYN and McCREADY 1941). Furthermore, ZECHMEISTER and TÓTH (1936) have shown that alcohol extraction of the initial material brings about that subsequent treatment of the cells with alkali breaks down the glucan. Finally, SEVAG, CATTANEO and MAIWEG (1935) have isolated from yeast a water-soluble polyose having a serological similarity to the capsule polysaccharides from *Pneumococcus*, type II (cf. also WHITE 1938).

The analytical glycogen values are presumably unreliable even if, after alkali treatment, the samples are precipitated with FEHLING's solution or if the yeast is extracted with alcohol prior to alkali treatment. Earlier methods of glycogen determination were modified for yeast by MAYER (1923). This author also presents an excellent review of the literature regarding the development of the methods. Attempts by the present author to determine the glycogen content of different yeasts by this method have not, however, yielded satisfactory results. The values obtained are usually much too high. The data in the literature regarding the glycogen content of yeast must likewise be suspected to be too high. Thus, in an account of the glycogen content of yeast, LÜERS (1929) states that, in a top yeast, the glycogen content after the consumption of glucose may amount to 39 % of the dry weight of the yeast.

In the following experiments, the relative quantities of glycogen in different yeasts were estimated only by treating the cells with iodine solution. On such treatment, the glycogen assumes a red-brown colour. STIER and NEWTON (1939) applied the iodine reaction as a quantitative measure of the glycogen content in yeast. These authors measured photoelectrically at 685 m μ the reflection of the iodine stained cells. When studying the glycogen formation in yeast, the present author (BRANDT 1941 b) estimated visually the occurrence of glycogen by means of microscopical ob-

servations of the cells stained with iodine. Likewise, MUSFELD (1942) has made use of this method in investigating the formation of glycogen during the uptake of sugar by yeast.

After the smeared preparation of the cells had been treated with iodine solution (LUGOL's solution) and immersed into a gum arabic solution containing iodine, the glycogen was seen in red-brown colour against the background of the yellow protoplasm. The presence of so-called glycogen vacuoles frequently described in early literature could not be observed. They presumably do not exist, since they appear neither in visible light, ultraviolet light, nor in dark field illumination in living cells which, to judge from the iodine reaction, contain large quantities of glycogen.

After cells of baker's yeast are stained with iodine, only a small amount of glycogen becomes visible. In fed yeast, however, the cells exhibit a very strong glycogen reaction. In starved yeast no glycogen can be demonstrated. Budding yeast yields a clearly positive reaction with iodine. The amount of glycogen in this yeast, however, is considerably smaller than that in fed yeast. If during pretreatment of baker's yeast ammonium chloride is replaced by asparagine, which has long been known as an excellent nitrogen source for yeast, this growing yeast also shows a lower glycogen content than that fed with glucose alone. This holds true despite the fact that glucose is present in moderate excess during the experiment. An interpretation of these phenomena will be found below. These observations were supported by fixing the cells with calcium picrate and, after treatment with 4 % chromic acid solution, staining the glycogen with fuchsine sulphurous acid by the method of SCHABADUSCH (1937).

Apart from glycogen, the non-reducing disaccharide trehalose is the most important reserve carbohydrate of baker's yeast (cf. KOCH and KOCH 1925, TANRET 1931, KLUYVER and VAN ROOSMALEN 1932, STEINER and CORI 1935, and MYRBÄCK 1936). In agreement with MYRBÄCK, BRANDT (1941 a) observed that 7—9 % of the dry weight of Swedish baker's yeast consisted of trehalose.

In brewer's yeast, trehalose was only detected in small amounts in the form of monophosphoric ester, which is obtained after fermentation of sugar by this yeast (ROBISON and MORGAN 1928, VEIBEL 1931).

Despite the fact that baker's yeast contains large quantities of trehalose, this, in contrast to glycogen, is fermented but slowly in the intact cell. After the cells are dried, or under the influence

of certain cell poisons, a more rapid break-down of the cellular trehalose is observed (MYRBÄCK and ÖRTENBLAD 1936, 1937 a and b). Supposing that trehalose is present in the cell as reserve carbohydrate, the break-down of which is mainly associated with processes other than those occurring in the stationary endogenous metabolism of yeast, BRANDT (1941 a) has studied the behaviour of trehalose during the growth of yeast. These investigations have shown that in yeast suspended in a salt solution, free from nitrogen compounds but containing cane-sugar, the latter is partly stored as trehalose (cf. also STEINER and CORI). On the other hand, in the presence of ammonium sulphate as a nitrogen source, the trehalose present in the initial yeast is largely broken down.

Analyses of different mother yeasts proved a deficiency of trehalose in the alcohol-forming Z 2 and Z 3 yeasts which had been slightly aerated during cultivation (< 0.25 % trehalose of the wet weight of the yeast). However, the Z 4 yeast, cultivated under strongly aerobic conditions, contained trehalose to somewhat less than 1 % of the wet weight of the yeast. In baker's yeast (Z 5), which like the preceding yeasts had been strongly aerated, a very high trehalose content (c. 2 %) was found. This may be attributed to the excess of sugar relative to added nitrogen, which prevailed at the end of the incubation. After heat-treatment of the different yeasts at 50° C. for 5, 10, and 15 minutes, the intensity of the endogenous respiration of the trehalose-containing yeasts was considerably increased. The analyses revealed a simultaneous break-down of part of the trehalose in the cells.

From the above observations it results that trehalose, which is slowly broken down during endogenous metabolism of the intact cell under aerobic conditions, may serve as an energy source in growth processes or in the restitution of any injured cells. The break-down of trehalose can be observed, for example, when a suspension of dried yeast is shaken under aerobic conditions; the majority of the properties of the yeast are then restituted (cf. below). A similar break-down can be seen during the experiments on heat-treated cells. Growth processes seem to inhibit the storage of trehalose. Its formation, however, may probably be dependent on simultaneous fermentation and on the degree of ripeness of the yeast. No estimations of the trehalose contents in the pre-treated yeasts were performed. Nevertheless, it can be regarded

as proved that fed yeast contains more and budding yeast less trehalose than the initial baker's yeast. The trehalose content of starved yeast is probably somewhat lower than that of baker's yeast.

3. Respiration and Fermentation.

Manometrical measurements of the endogenous respiration and fermentation in pretreated yeasts are illustrated in Figs. 11 and 12. Fig. 11 gives an example of the uptake of oxygen and the production of carbon dioxide in air. The value of the respiratory quotient (CO_2/O_2) in the yeasts investigated — with the exception of starved yeast — is only slightly below 1, thus indicating that the oxidized substrates are carbohydrates (cf. STIER and STANNARD 1936, and BOREI 1942). The endogenous respiration of Swedish baker's yeast has been studied in greater detail by BOREI. In his paper, examples are given of the relatively great variations in the respiration intensity of baker's yeast from different seasons of the year. BOREI has shown that the respiration occurring over the system cytochrome oxidase — cytochrome can be resolved into two processes: firstly, a monomolecular, rather rapidly ceasing respiration, the rate of which depends on the concentration of the substrate and, secondly, a respiration occurring at a constant rate, possibly limited by the concentration of some participating enzyme. These latter investigations were performed on baker's yeast of the earlier type A. The magnitude of Q_{O_2} ($\mu\text{l. of oxygen consumed per hour per mg. of dry weight}$) is in good agreement with the value for the more recently cultivated type B. The value of the respiratory quotient of type A, however, derived from measurements at pH 5, is somewhat higher than 1. (See BOREI and LINDWALL (1943) who demonstrated that the endogenous, constant respiration was dependent on the pH of the suspension medium.)

In Chapter IV are described determinations of the amounts of carbon dioxide bound in the different yeasts; the magnitude of respiration and fermentation taking place at the time of fixation of the yeast with acid corresponds approximately to the metabolism occurring in the period 30—45 minutes in Figs. 11 and 12.

A comparison of the magnitude of endogenous metabolism taking place in pretreated yeasts under aerobic conditions with that in baker's yeast gives the following results (Fig. 11). When

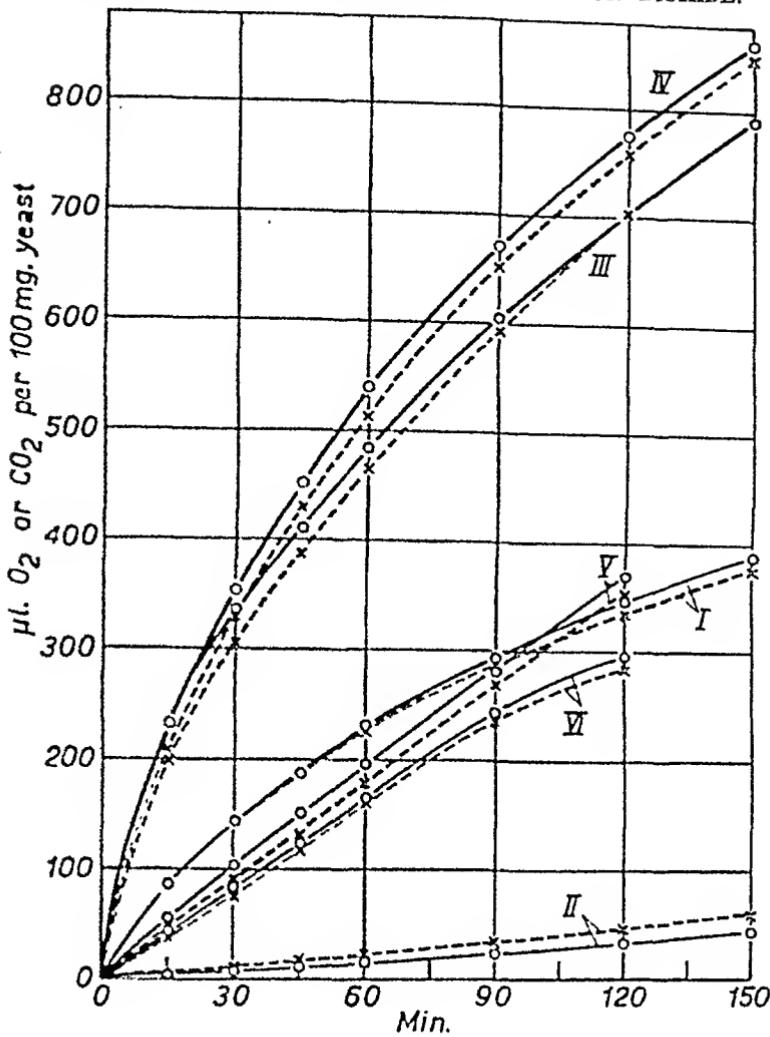


Fig. 11. Endogenous aerobic metabolism of different yeasts. Summation curves.
 — O₂-consumption. - - - CO₂-formation.
 (I) Baker's yeast. (IV) Budding yeast.
 (II) Starved yeast. (V) Dried yeast (unwashed).
 (III) Fed yeast. (VI) " " (washed).

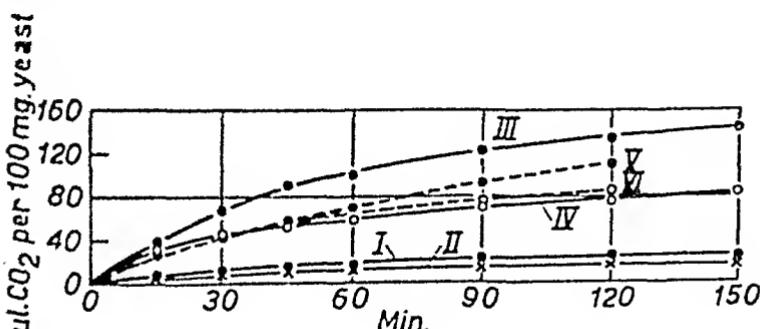


Fig. 12. Endogenous anaerobic metabolism of different yeasts. Key as in Fig. 11.

the yeast is starved, the monomolecular respiration disappears, largely owing to the consumption of glycogen, while only a very small, rather constant respiration remains (cf. curves I and II). The value of the respiratory quotient is remarkably high and decreases continuously in the course of the experiment from about 1.9 to 1.4. Both fed and budding yeasts exhibit considerably increased respiration intensities (curves III and IV). According to STIER and STANNARD (1936), the intensity of the endogenous respiration is almost proportional to the glycogen content of the cells. In the experiments described above, however, the fed yeast contains considerably more glycogen than the budding yeast. In spite of this, the respiration intensity of fed yeast, rich in glycogen, is somewhat lower than that of budding yeast. In the respiration of the latter, apart from glycogen, part of the trehalose is also presumably oxidized in connection with growth processes.

Dried yeast, stored in the refrigerator for 13 months before its use, showed rather strong and constant respiration throughout the experiment (curve V). For purposes of comparison with the values for the other yeasts, the respiration is calculated per gram of wet weight. A marked difference is observed if the dried yeast is washed twice with water in the centrifuge immediately before measurement. The respiration then becomes somewhat lower towards the end of the experiment (curve VI). This phenomenon may be attributed to a markedly increased permeability of the yeast after drying, resulting in the migration of numerous compounds from the cells, such as inorganic phosphate, phosphate esters, cozymase, and other nitrogen-containing substances (NILSSON and ELANDER 1941, BRANDT 1942 a, and RUNNSTRÖM, BRANDT and MARCUSE 1943). Trehalose must presumably be regarded as the main substrate for the considerable endogenous respiration, just as for the relatively high endogenous fermentation (cf. Fig. 12). Trehalose is exploited as an energy source for the restitution processes taking place in suspensions of dried yeast.

In contrast to brewer's yeast, the cells of baker's yeast are not killed during drying. When dried baker's yeast is transferred into a nutrient solution, the cells begin to develop buds. Frequently, however, their cleavage is abnormal. Investigations of the metabolism of an exogenous substrate by dried yeast shows that the respiratory system of dried yeast is markedly inhibited. With increasing time of storage, a progressive decrease in the respiratory power is observed, and aerobic fermentation increasingly domi-

nates the respiration, while the total metabolism of glucose decreases (see RUNNSTRÖM, BRANDT and MARCUSE 1943). When the dried cells are shaken under aerobic conditions in water or in buffer overnight, the initially high permeability of the dried yeast decreases. Addition of glucose during pretreatment of the dried yeast contributes considerably to the restitution of the normal semi-permeability of the cells. A change in the physiological character of the dried cells appears most clearly; after shaking under aerobic conditions the cells assume more and more the properties of fresh baker's yeast (RUNNSTRÖM and SPERBER 1938 b, RUNNSTRÖM and BRANDT 1941, BRANDT 1942 a, and RUNNSTRÖM, BRANDT and MARCUSE 1943).

Fig. 12 illustrates the carbon dioxide formation in different yeasts measured under anaerobic conditions. Both in baker's yeast and in starved yeast this is extraordinarily small, while in the other yeasts endogenous fermentation is clearly observed. Its intensity, however, never reaches the high values found in endogenous respiration. Under anaerobic conditions, in contrast to its behaviour under aerobic conditions, fed yeast rich in glycogen exhibits a fermentation remarkably higher than that of budding yeast.

Investigations of the respiration, aerobic fermentation, and glucose uptake during pretreatment of baker's yeast with glucose or glucose + ammonium chloride are well suited to elucidate the above observations regarding the changes in the content of reserve carbohydrates in the yeasts. ZELLER (1926) has shown that the presence of ammonium chloride promotes considerably the turnover of glucose. This evidence has later been confirmed by SMYTHE (1939) who, in experiments performed both under anaerobic conditions (gaseous phase with carbon dioxide) and in air, found that glutamine, asparagine and, to a somewhat smaller extent, arginine increased fermentation (see also KÖGL and BORG's (1941) studies of the factor Z, discovered by v. EULER, and their review of the work in this sphere). MEYERHOF and IWASAKI (1930) have further demonstrated that the turnover of sugar in low concentration, in the presence of ammonium chloride, is accompanied by an obvious increase in the respiration of the yeast. Using Swedish baker's yeast, RUNNSTRÖM, BRANDT and MARCUSE (1940) have confirmed these observations. In the following description of the metabolism of glucose under aerobic conditions, both in the presence and absence of ammonium chloride, it is intended to

give a short review of certain characteristic reactions occurring in connection with respiration and fermentation during pretreatment of the yeast.

Fig. 13 shows the manometrically measured oxygen consumption and carbon dioxide production in baker's yeast consuming

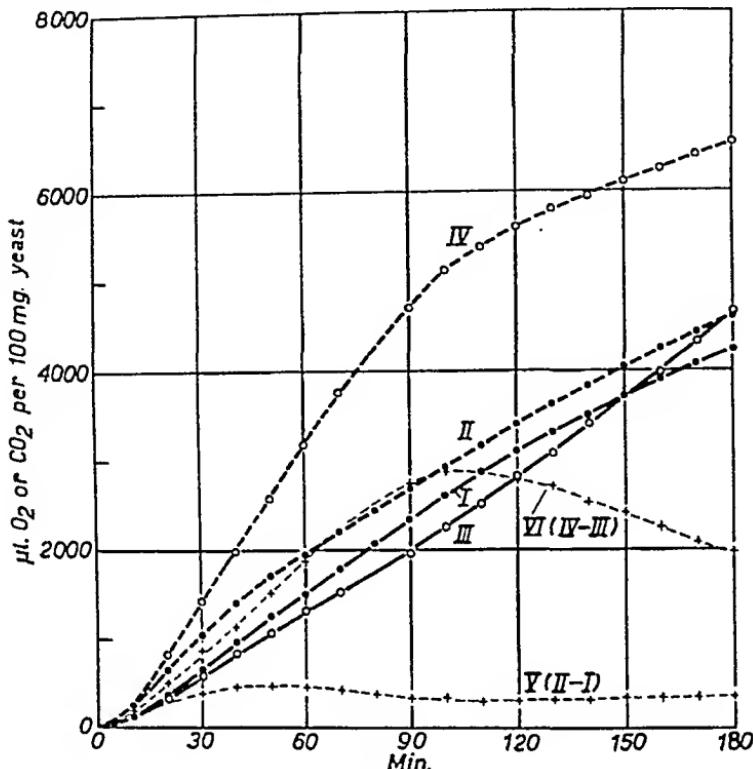


Fig. 13. Respiration and fermentation during aerobic metabolism of glucose with and without addition of NH_4Cl . Initial concentration per sample of 2.4 ml.: 20 mg. baker's yeast, 5.0 mg. glucose, 0.45 mg. NH_4Cl in 0.08 M Na-SS buffer.

Summation curves.

- (I) O_2 -consumption, and (II) CO_2 -formation during metabolism of glucose.
- (III) " " " (IV) " " during metabolism of glucose + NH_4Cl .
- (V) Fermentation- CO_2 during metabolism of glucose.
- (VI) " " " " " + NH_4Cl .

glucose, with and without addition of ammonium chloride. The yeast employed was of the type A. Initial concentration of baker's yeast 0.83 %, glucose 0.0115 M, and ammonium chloride 0.0035 M. The volume of the mixture was 2.40 ml., containing 20 mg. yeast, 5.0 mg. glucose, and 0.45 mg. ammonium chloride.

From curves I and III it results that the respiration is weakly inhibited in the beginning of the experiment in the presence of ammonium chloride, increasing after the lapse of about 90 minutes

to a value above that obtained with a substrate consisting only of glucose. If the value of the respiratory quotient (CO_2/O_2) is assumed to be 1, the difference between the values of the total amount of carbon dioxide developed (cf. curves II and IV) and respiratory carbon dioxide (= amount of oxygen consumed) is equal to the fermentation carbon dioxide (see SPERBER and RUNNSTRÖM 1939). The corresponding curves are drawn in the figure (curves V and VI). From these curves it is evident that, in the presence of glucose alone, only weak aerobic fermentation is obtained, while the presence of ammonium chloride considerably increases the aerobic fermentation. With the concentration of glucose and ammonium chloride in question, the fermentation ceases after about 100 minutes. The same observations were made in experiments on baker's yeast, type B.

In analyses of the alcohol produced in the presence of ammonium chloride, 80—90 % of the amount of alcohol calculated from the fermentation carbon dioxide produced may be recovered prior to the increase in respiration and the decrease in carbon dioxide formation. Table 5 gives the amounts of alcohol determined in three experiments performed in flasks with and without potassium hydroxide in the central tube. In order to obtain sufficient quantities of alcohol at the end of the experiment, the initial concentration of glucose was — in contrast to the preceding experiment — 10 mg. per volume of suspension in the flask. 150 minutes after addition of glucose and ammonium chloride the amount of alcohol formed was estimated. At this time, the aerobic fermentation had not yet ceased. The amounts given are calculated in μmol . per 20 mg. of yeast.

Table 5.
Estimation of alcohol produced during aerobic fermentation.

Ex- periment	Fermentation- CO_2 obtained in the pre- sence of NH_4Cl $\mu\text{mol.}$	Alcohol in flask with KOH		Alcohol in flask without KOH	
		Amount estimated $\mu\text{mol.}$	Percentage of the amount calculated from fermenta- tion- CO_2	Amount estimated $\mu\text{mol.}$	Percentage of the amount calculated from fermenta- tion- CO_2
1	51	44	86	49	96
2	42	32	76	39	93
3	41	32	78	39	95

It is seen from Table 5 that in the flasks without potassium hydroxide in the central tube more alcohol was recovered than in those with potassium hydroxide. This may be related to the observations described in Chapter III, *viz.* in the presence of large amounts of carbon dioxide the metabolism of glucose is greater than normal. At the end of the experiment, the flasks without alkali contain about 10 % carbon dioxide in the gaseous phase owing to the high carbon dioxide formation in the presence of ammonium chloride. In experiments in which such high carbon dioxide production takes place, the turnovers in the flasks with and without potassium hydroxide in the central tube are in all probability not quite equal. This involves a certain error in the calculations of the respiration and fermentation curves. To judge from experiments not described in this paper, performed with varying concentrations of yeast and glucose, this error does not influence the principal characteristics of the respiration and fermentation curves.

Despite the above considerations, suspensions with relatively high metabolism have also been employed in the experiments described below; this proved to be necessary for experimental reasons. By employing rather high concentrations of yeast or substrate, the volume of suspension could be decreased to a magnitude appropriate to the culture flasks available. Under these conditions, the material was sufficient for estimations of the changes within the substrate and the cell contents at different periods. The manometrical determinations were performed only in order to check the magnitude and the time of the deviations in the course of respiration and fermentation.

Investigations by WERTHEIMER (1934) on baker's yeast, by MCANALLY and SMEDLEY-MACLEAN (1935) and WILLSTÄTTER and ROHDEWALD (1937) on brewer's yeast (see also the literature cited in these papers) showed that glucose disappeared more rapidly than corresponded to its consumption by respiration and fermentation. The old observation was thus confirmed that glucose in the cells is built up to higher carbohydrates. The consumption of glucose corresponding to the amounts of respiration and fermentation carbon dioxide, which were measured before the end of aerobic fermentation, *i. e.* before the oxidation of the products formed during fermentation had begun, may be calculated. By subtracting the calculated amounts of glucose from the total glucose consumption evaluated by simultaneous glucose analyses,

a measure is obtained of the amount of glucose consumed in the synthesis of higher carbohydrates and intermediates. The calculation is based upon the assumption that the respiratory quotient is 1 (1 mol. respiration carbon dioxide thus corresponding to $\frac{1}{6}$ mol. glucose, and 1 mol. fermentation carbon dioxide to $\frac{1}{2}$ mol. glucose).

In consequence of the respiration and the strong aerobic fermentation, a higher glucose consumption is obtained in the presence of ammonium chloride than in the absence of the nitrogen source. The following estimations of the consumption of glucose and the magnitude of carbohydrate synthesis are in complete agreement with the observations described earlier regarding the differences in the amount of glycogen and trehalose in fed and budding yeasts.

In the above described experiments with an initial concentration of 5.0 mg. of glucose per sample, the glucose consumption was calculated from the values of respiration and fermentation carbon dioxide after 90 minutes to be 2.50 mg. in the presence of ammonium chloride, and 0.94 mg. if only glucose was consumed. Determinations carried out after 90 minutes showed that the total consumption of glucose (determined according to the method of WILLSTÄTTER and SCHUDEL 1918, modified according to GOEBEL 1927) amounted to 4.20 mg. and 3.40 mg. in the presence and absence of ammonium chloride, respectively. Hence, 41 % of the total amount of metabolized sugar was used in the synthesis (including that of intermediate products) during the turnover of glucose in the presence of ammonium chloride, while the corresponding value in the turnover of glucose alone amounted to 72 %. At the end of the aerobic fermentation, the glucose concentration, in the presence of ammonium chloride, had decreased to about 16 % of the initial concentration. The alcohol formed, however, was accumulated to a considerable excess relative to the glucose present. Only after the concentration of glucose had further decreased was the alcohol oxidized by respiration. SPERBER and RUNNSTRÖM (1939) found that, in the presence of even small amounts of glucose, alcohol was not affected by baker's yeast. Unfortunately, their determinations of alcohol were performed according to WIDMARK's method, which later proved to be less reliable in work on yeast. The present alcohol estimations nevertheless lend considerable support to their observations.

In the presence of ammonium chloride, part of the glucose is

used for the synthesis of protein; hence only part of the sugar is stored in the cells as reserve carbohydrates (see also below, in connection with the changes in the dry weight of the yeast after different pretreatments). From estimations of the uptake of nitrogen it became obvious that after 90 minutes about 60 % of the amount of nitrogen added had been taken up by the cells. These determinations were performed on samples from flasks which had been shaken with and without potassium hydroxide in the central tube. It was observed, just as in the recently described estimations of the alcohol formed, that the uptake of nitrogen occurred more rapidly in the absence of alkali, a phenomenon which supports the view that the aerobic metabolism is activated by carbon dioxide (for further details, see Chapter III).

Further measurements of the respiration and fermentation and of the uptake of glucose, with baker's yeast of the same type as that used in the examples reported above, though with other concentrations of glucose and ammonium chloride, may be found in RUNNSTRÖM, BRANDT and MARCUSE (1940). In connection with their experiments on the assimilation of ammonia, these investigators showed that storage of higher carbohydrates in yeast was impeded by the presence of ammonium chloride not only under aerobic, but also under anaerobic conditions. The effect of ammonium chloride on the metabolism of glucose in baker's yeast was decidedly smaller under anaerobic than under aerobic conditions, a fact which may be related to the slower uptake of nitrogen in the former case. Ammonia nitrogen taken up by the cells was found to be used almost wholly for the synthesis of protein.

According to a private communication, RUNNSTRÖM has found that the increasing effect of ammonium chloride on aerobic and anaerobic fermentation first appears at higher concentrations of glucose. The aerobic fermentation increases with increasing concentration of glucose. With lower concentrations, however, a pronounced increase in respiration is observed, this observation being in good agreement with the findings of MEYERHOF and IWASAKI (1930).

The investigations described in the next sections deal with the changes in acid-soluble nitrogen and both free and bound acid-soluble phosphate in the cells during the consumption of glucose in the presence and absence of ammonium chloride. In these experiments, the yeast concentration in the suspensions amounted

to 1.67 %, the initial concentration of glucose being 0.0115 M and that of ammonium chloride 0.0035 M in 0.08 M Na-SS buffer. For purposes of comparison with the courses of respiration and fermentation exhibited in Fig. 13, Fig. 14 shows corresponding courses found when 40 mg. of yeast per sample were used instead of 20 mg., but the same glucose and ammonium chloride concentrations as in the previous experiments.

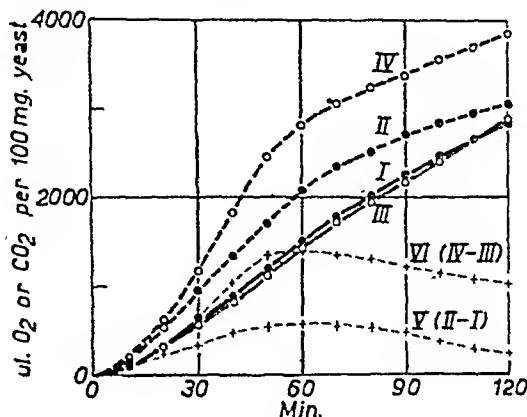


Fig. 14. Respiration and fermentation during aerobic metabolism of glucose with and without addition of NH_4Cl . Initial concentration per sample of 2.4 ml.: 40 mg. baker's yeast, 5.0 mg. glucose, 0.45 mg. NH_4Cl in 0.08 M Na-SS buffer. Summation curves. Key as in Fig. 13.

The shapes of the curves in the two experiments are in good agreement. The initial depression in the respiration appears, however, less pronounced than usual in the latter case. Owing to the small amount of sugar used in relation to the yeast concentration, the aerobic fermentation already decreases after about 60 minutes.

4. Nitrogen Metabolism.

For determinations of the amount of ammonia present in the suspension medium and of the content in the cells of total nitrogen soluble in 4 % trichloroacetic acid at different periods, the suspensions were shaken in 1.8 litre culture flasks containing 120 ml. suspension of the composition described above. When the yeast is shaken in buffer without addition of glucose or ammonium chloride, the content of acid-soluble nitrogen within the cells changes but insignificantly (Table 6). The values reveal that the amounts present in starved yeast are practically equal to those in the initial baker's yeast.

Table 6.

Content of acid-soluble nitrogen in cells of baker's yeast after aeration.

Time of shaking in hours	mg. acid-soluble N per g. yeast
0	3.22
1	3.11
3	3.04
5	3.11
23	3.25

The uptake of nitrogen in the presence of ammonium chloride and the changes in the cell content of acid-soluble nitrogen during the consumption of glucose and glucose + ammonium chloride are seen from Fig. 15. This figure includes data from two experiments in which the two yeasts exhibited a pronounced difference in their initial contents of total acid-soluble nitrogen. It is evident from the curves, however, that similar changes in the acid-soluble nitrogen fractions occurred in the two cases.

The initial contents of total nitrogen in the yeasts used in experiments I and II amounted to 17.9 and 16.8 mg. nitrogen per g. yeast. Of this quantity, about 1 mg. consisted of amide-nitrogen. The amount of ammonia nitrogen in the yeast was extremely small, *viz.* only about 2—3 μ g. per g. yeast. It should be emphasized here that when suspensions of baker's yeast were shaken without addition of substrate, say for 3 hours, no uptake of added ammonium chloride by the cells could be detected. According to unpublished investigations by SPERBER at this Institute, this phenomenon might be due to the relatively high initial total nitrogen content of the yeast. When the yeast was shaken in the absence of substrate, no liberation of nitrogen was found.

Curve A in Fig. 15 shows that the ammonia added (corresponding to 3.00 mg. nitrogen per g. yeast) was taken up completely by the yeast within less than 90 minutes. In the consumption of glucose without addition of ammonium chloride (curves I a and II a), the amount of acid-soluble nitrogen is decreased during the time of the experiment. In the presence of ammonium chloride (curves I b and II b), however, an increase in the content of acid-soluble nitrogen is observed in the beginning, simultaneously with the high uptake of ammonia. Later, the value decreases rapidly, in-

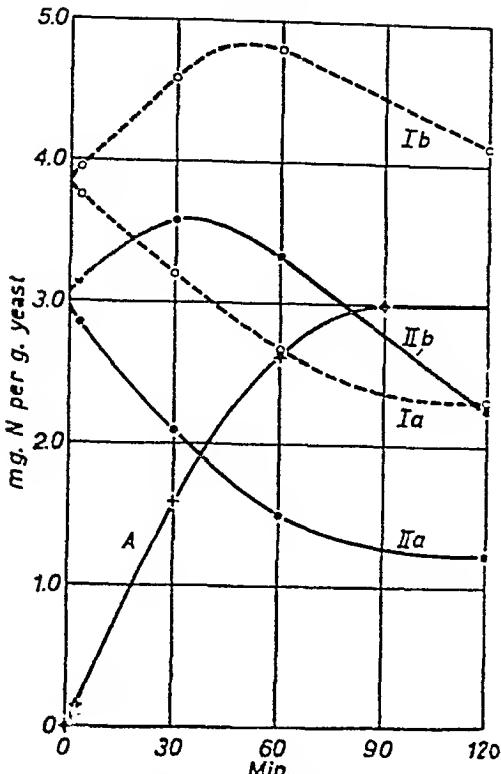


Fig. 15. Uptake of nitrogen and changes in the content of acid-soluble nitrogen during aerobic metabolism of glucose with and without addition of NH_4Cl . Initial concentrations of yeast, glucose, and NH_4Cl correspond to those in Fig. 14. I and II: Experiments on yeast with different initial contents of acid-soluble nitrogen. (A) Uptake of ammonia-nitrogen in the presence of NH_4Cl . (Ia) and (IIa) Changes in the acid-soluble nitrogen during consumption of glucose. (Ib) and (IIb) • • acid-soluble nitrogen during consumption of glucose + NH_4Cl .

dicating that the ammonia is incorporated into acid-insoluble compounds. Analogous observations were made by RUNNSTRÖM, BRANDT and MARCUSE (1943) when investigating the restituting effect of shaking on dried baker's yeast under aerobic conditions.

5. Phosphate Distribution.

Using the same concentrations of yeast and the same additions as in the preceding experiments, the changes in the content of "free" and bound phosphate (soluble in 4 % trichloroacetic acid) were estimated during the shaking of the yeast under aerobic conditions without addition of substrate and with addition of glucose and glucose + ammonium chloride (BRANDT 1942 b). The

experiments were performed in 1.8 litre culture flasks initially containing 180 ml. suspension. The yeast used was of type A. The results are given in Fig. 16. The curves represent the mean values obtained in numerous experiments embracing about 700 phosphate determinations. The average total phosphate content of the yeast was 3.70 mg. phosphorus per g. yeast, corresponding to 120 μmol . orthophosphate. The phosphate soluble in trichloroacetic acid, which was estimated without preliminary hydrolysis, will be denoted as "free phosphate". The corresponding curves in the figure are marked with a. Part of this phosphate may originate from amounts of 1,3-diphosphoglyceric acid and acetyl-phosphate present, containing phosphate loosely bound to the carboxyl groups (see NEGELEIN and BRÖMEL 1939, LIPMANN 1941, and LYNNEN 1941). Bound acid-soluble phosphate was calculated as the difference between the total acid-soluble phosphate and the free phosphate. If the bound acid-soluble phosphate was hydrolysed in normal sulphuric acid for 7 minutes at 100° C., 40—45 % of the phosphate was split off. Although the change in the total amount of bound acid-soluble phosphate varies remarkably during the experiment, approximately the same percentage of bound phosphate is always hydrolysed. The greater part of the hydrolysed phosphate probably consists of pyrophosphate.

Curves I a and I b in Fig. 16 (heavy lines) illustrate the changes in the amount of free and bound acid-soluble phosphate in the cells during shaking of the yeast without addition of substrate. In the beginning, the amount of free phosphate (I a) decreases somewhat, increasing later to an almost constant level. The content of bound acid-soluble phosphate (I b) at first increases very markedly during shaking. This change, which is already pronounced after some minutes, causes a remarkable variation in the initial analytical values. The time between the preparation of a suspension and its fixation thus greatly affects the size of this phosphate fraction, but it also influences the changes in the phosphate fractions in experiments upon addition of substrate. All curves drawn with thin lines exhibit schematically the alterations in the phosphate fractions for yeasts with an initially high content of bound acid-soluble phosphate, obtained by shaking the suspensions for about 1 hour before the start of the experiment.

In the aerobic consumption of glucose by yeast, a large part of the free phosphate (II a) is bound within the first minutes after the addition of sugar. At later stages, a low content of free

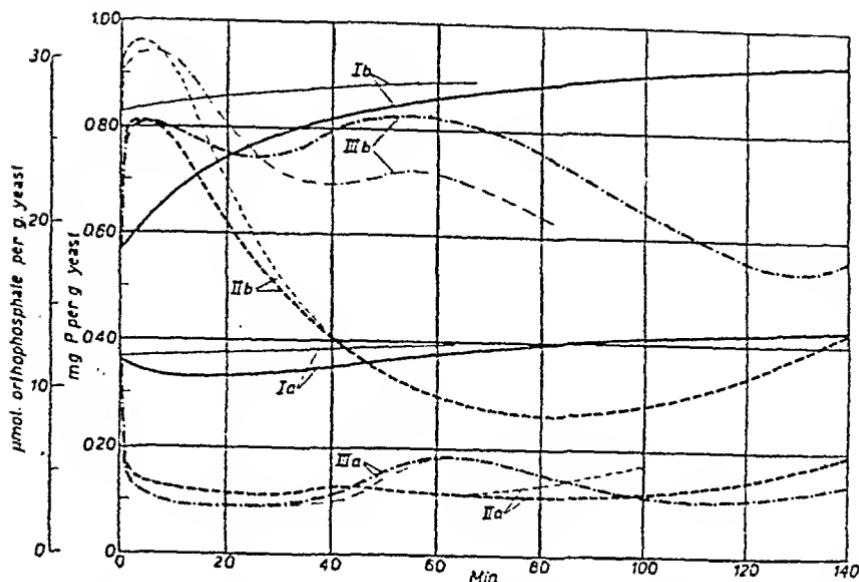


Fig. 16. Changes in the content of free phosphate and bound acid-soluble phosphate during aerobic shaking of baker's yeast and consumption of glucose in the presence and absence of NH_4Cl . Initial concentrations of yeast, glucose, and NH_4Cl correspond to those in Fig. 14. The thin lines schematically represent changes occurring when yeasts are used which have initially high contents of bound acid-soluble phosphate.

- (I a) Changes in the free phosphate during agitation without added substrate.
- (II a) * * * * * metabolism of glucose.
- (III a) * * * * * metabolism of glucose + NH_4Cl .
- (I b) * * * bound acid-soluble phosphate during agitation without added substrate.
- (II b) * * * * * during consumption of glucose.
- (III b) * * * * * during consumption of glucose + NH_4Cl .

phosphate is maintained within the cells, which increases again slightly after the glucose is completely consumed. In the beginning, the amount of bound acid-soluble phosphate (II b) increases rapidly, decreasing again after less than 10 minutes to a value below the initial content of this phosphate fraction. This phosphate seems to have been incorporated into acid-insoluble compounds by way of intermediate acid-soluble materials.

Analogous observations were made by LYNEN (1941) (see also MIRSKI and WERTHEIMER 1939) who showed that, in the aerobic consumption of alcohol, acetaldehyde and acetate by moderately starved yeast, the content of free phosphate decreased within the protoplasm. LYNEN also found that when alcohol was added the phosphate was incorporated into the acid-insoluble compounds, since the total content of acid-soluble phosphate decreased. He

showed, moreover, that the amount of free phosphate disappearing in the consumption of glucose was remarkably larger under aerobic than under anaerobic conditions.

In the turnover of glucose in the presence of ammonium chloride, the free phosphate is bound very rapidly (III a), as is the case in the absence of the nitrogen source. The content of free phosphate within the cell hereby decreases to a lower value than that observed in the consumption of glucose alone. After about 40 minutes, the amount increases to a maximum, subsequently decreasing again. After the consumption of the substrate the amount of free phosphate increases slowly. The cause of the maximum at about one hour after the start of the experiment is unknown. An interruption of aerobic fermentation may contribute to the temporary increase in the content of free phosphate within the protoplasm; when the oxidation of alcohol commences, the phosphate content again decreases slowly.

The change in the content of bound acid-soluble phosphate during glucose metabolism in the presence of ammonium chloride (III b) varied very markedly in different experiments. Curve III b exhibits a course completely different from that of the corresponding curve II b. During the first minutes, as in the absence of ammonium chloride, a strong increase in the amount of bound phosphate was obtained, which decreased after some time, ultimately increasing anew to a second maximum. In experiments on yeasts with an initially high content of this phosphate fraction, the latter maximum could frequently not be observed; the phosphate content, however, remained rather constant for some time. The maximum in the free phosphate content obtained about one hour after the start of the experiment thus apparently corresponds to the changes in the content of bound acid-soluble phosphate exhibited in the curve, indicating that a change takes place in the metabolic processes associated with phosphorylation.

When the substrate is exhausted, the contents of free and bound acid-soluble phosphate approach the values typical of the yeast shaken without substrate.

According to LYNEN (1941), the content of free phosphate within the protoplasm is associated with PASTEUR's reaction. LYNEN believes that the competition between the respiration and fermentation processes for the inorganic phosphate may explain this reaction (cf. JOHNSON 1941). Under aerobic conditions, fermentation is assumed to be suppressed, while in the respiration

processes a more rapid decrease in inorganic phosphate is obtained than during the fermentation processes. Consequently, the liberation of free phosphate through dephosphorylation processes, which according to LYNEN occur at an unchanged rate, cannot take place rapidly enough to maintain the concentration of free phosphate necessary for a strong fermentation, this free phosphate being bound during the dehydrogenation of triosephosphate (WARBURG and CHRISTIAN 1939). LYNEN thus supposed strong fermentation to be associated with a high content of free phosphate within the protoplasm.

In the experiments described above, during aerobic consumption of glucose in the presence of ammonium chloride (III a), the content of free phosphate decreased during the period of aerobic fermentation to a smaller extent than was found in the consumption of glucose alone (II a). This holds, despite the fact that in the former case the aerobic fermentation is remarkably stronger than in the latter and that, apart from this, the respiration is unchanged or but slightly checked in the presence of ammonium chloride. The consumption of glucose in case III, moreover, is higher than in case II, which corresponds to the behaviour during anaerobic fermentation. From the above data it results that an increased fermentation may occur without an increased content of so-called free phosphate in the protoplasm when a growth metabolism takes place.

6. Alterations in Dry Weight.

During pretreatment of baker's yeast with the aim of obtaining starved, fed and budding yeasts the dry weight of the initial yeast is changed. In the following section, such alterations are considered which are found when baker's yeast is pretreated under the conditions described above for the preparation of such yeasts. When the yeast is starved overnight, the dry weight is decreased by c. 3.5—4 % of the initial dry weight. During the first 3 hours, the dry weight already decreases by more than 1 %. The decrease can chiefly be attributed to the consumption of carbohydrates occurring during endogenous metabolism. According to GENAUD (1929), shaking of the yeast causes practically no loss of the cell constituents into the suspension medium.

After feeding, the amount of dry substance increases by about 39 % of the initial dry weight. This increase corresponds to a

storage of reserve carbohydrates (see above) amounting to about 26—27 % of the glucose added during pretreatment. In the presence of ammonium chloride, the increase in dry weight in the budding yeast is only c. 28 % of the initial dry weight. Estimations of the amount of nitrogen taken up during the pretreatment showed that after $2\frac{1}{2}$ hours 60—65 % of the nitrogen added was consumed. If the dry weight of the initial yeast is put at 26 %, and the nitrogen taken up is assumed to be principally employed in the synthesis of "raw protein" containing c. 16 % nitrogen (according to CLAASEN 1934), then 13—14 % of the initial dry weight is the maximum increase that may originate from the formation of protein. During pretreatment of the yeast with glucose in the presence of ammonium chloride, approximately half of the total increase in the amount of dry substance may thus be supposed to originate from the formation of acid-insoluble nitrogen compounds, while the other half consists of reserve carbohydrates formed. On the basis of the above assumptions, the additional supply of stored carbohydrates in the budding yeast is only one third of that obtained in the yeast fed with glucose alone.

When not otherwise stated, the quantities of acid-labile bound carbon dioxide in the yeasts pretreated in different manners are expressed per gram of initial wet weight of baker's yeast. The alterations in dry weight of the yeast during pretreatment are not considered in the calculations. When the values for the different yeasts are compared, figures calculated per gram of dry weight are probably less comparable than those expressed per gram of initial wet weight. It is improbable that the amount of reserve carbohydrates stored in fed and budding yeasts greatly affects the amount of plasma components capable of binding carbon dioxide.

It is difficult to correct accurately for the increase in wet weight due to protein formed during pretreatment under addition of ammonium chloride, since the fate of the water in yeast during pretreatment is not known in detail. The increase in the protein content of the yeast may be estimated to be 25—30 %. If the water content of the cell increases proportionally, the wet weight of the yeast should be about 15 % higher. As baker's yeast is treated in the suspension medium without addition of salts other than ammonium chloride, the total content of salts in the yeast cells after pretreatment must be the same per gram of initially

weighed yeast. As it is unknown in what way the quantities of salts or proteins in the cell affect the ability to bind carbon dioxide, the introduction of an approximate correction for the changes in wet weight obtained in budding yeast seems to be unjustified. No calculations were made of carbon dioxide bound per gram of dry weight or per unit weight of nitrogen of the initial baker's yeast, since these scarcely appear more advantageous than those described above. Variations in the dry weight of baker's yeast and in the content of total nitrogen are rather insignificant.

The content of intercellular water in the initial baker's yeast was calculated to approximate to 25 % of the wet weight (JUST 1940 and unpublished investigations on Swedish baker's yeast by MALM at this Institute). The yeasts were always washed by centrifuging the suspensions for 6 minutes in an "Ecco" centrifuge and immediately pouring off the supernatant liquid. After the last washing, the walls of the tube were dried with filter paper in order to remove possible residual water. When 2—10 g. of baker's yeast is treated in this manner, it is observed that the dry weight of the centrifuged yeast has decreased by about 12 % of the initial dry weight. This indicates that the intercellular water content is 32—33 % of the wet weight. In the preparation of yeast suspensions for the measurement of the amount of bound carbon dioxide, corrections for the changes in the amount of intercellular water after centrifugation of the yeast were not regarded as necessary. The content of intercellular water within the yeast was considered, however, in the calculation of the dilution water affecting the pH determinations in suspensions of heat-fixed yeast, since in these experiments rather concentrated suspensions were used (cf. Chapter V).

7. Characteristics of Pretreated Yeasts.

From the observations communicated above concerning the effect of different pretreatments on baker's yeast, it results that the pretreatments initiate characteristic changes in the composition and the properties of the initial yeast. The changes typical of the yeasts pretreated in different manners are listed in the following summary.

Starved Yeast. When baker's yeast is starved, the protoplasm is clearly vacuolized. The volutine grains rich in ribo-nucleotides

become aggregated. The small amount of glycogen present in the initial yeast has disappeared. The endogenous respiration has considerably decreased, simultaneously with a remarkable increase in the respiratory quotient. It should be mentioned here that during starvation the yeast probably loses part of its riboflavin, which may affect the oxidative processes in starved yeast (see STIER and SPRINCE 1941). MYRBÄCK and VALLIN (1944) have shown, moreover, that during aeration the aneurin in baker's yeast is transformed into aneurin disulphide. Under anaerobic conditions, the endogenous fermentation is, just as in baker's yeast, extremely weak. The amount of acid-soluble nitrogen in the cells is practically the same as in the initial yeast. The content of so-called free phosphate does not deviate noticeably from that in baker's yeast. The amount of acid-soluble bound phosphate, however, is greater in starved yeast.

Fed Yeast. In this type of yeast, the vacuoles have disappeared almost completely. The volutine grains are spread rather uniformly over the whole hyaloplasm. During pretreatment of baker's yeast with glucose, large amounts of glycogen and trehalose are stored in the fed yeast, and the dry weight increases significantly. The endogenous metabolism under both aerobic and anaerobic conditions is considerably increased. The content of acid-soluble nitrogen in the fed yeast is remarkably less than in baker's yeast. The amounts of acid-soluble free phosphate and bound phosphate within the cells are much lower than in the initial yeast.

Budding Yeast. This material exhibits quite well developed vacuoles. The volutine grains have begun to dissolve, and the occurrence of dissolved and partly new-formed nucleotides is clearly observed in the hyaloplasm. As a consequence of the consumption of glucose during pretreatment in the presence of a nitrogen source, an incipient budding is observed in the yeast together with a synthesis of protein, simultaneously with a strong aerobic fermentation of the glucose. The reserve carbohydrates are stored, though to a considerably smaller extent than in the preceding yeast. Despite this fact, the budding yeast shows a somewhat stronger endogenous respiration than the fed yeast. Under anaerobic conditions, the fermentation is lower than that of fed yeast, yet markedly higher than that of baker's yeast. The increase in dry weight is decidedly smaller than with the preceding

type of yeast. The content of acid-soluble free phosphate is of the same order of magnitude as in fed yeast, while the amount of bound acid-soluble phosphate is greater.

Dried Yeast. The cells are still alive after drying. The permeability to different substances, however, is markedly increased. Washing of the yeast with water therefore involves a loss of some low-molecular substances from the cell. The hyaloplasm is partly granular. After the dried yeast has been stored for a relatively short time, no glycogen is found in the cells. The endogenous metabolism of the yeast under aerobic conditions is of the same order of magnitude as in baker's yeast, though it does not decrease so rapidly. Under anaerobic conditions, the endogenous fermentation is considerably higher than in the initial yeast. The respiratory system is greatly affected by drying; a retardation of its function is observed to accompany an increasing domination of the anaerobic processes as the time of storage increases.

In Chapter III examples are given of the different contents of free phosphate in yeasts pretreated under aerobic and anaerobic conditions and in the presence of 25 % carbon dioxide in the gaseous phase. The data given refer to the conditions under which the determinations of the amount of acid-labile bound carbon dioxide were performed in different types of yeast.

CHAPTER III.

Effect of Carbon Dioxide on the Metabolism and Cell Constituents.

As, in the following chapters, experiments are described which were performed on yeast shaken with 0—50 % carbon dioxide in the gaseous phase, some preliminary experiments will be communicated in order to elucidate the effect of carbon dioxide on the yeast cells, *viz.* on the endogenous metabolism, on the free phosphate and ammonia nitrogen contents of the protoplasm and the uptake of glucose and pyruvic acid by the yeast.

Apart from the few observations, described in Chapter I, regarding the effect of carbon dioxide on the metabolism of yeast, no further data are available from the literature. Our knowledge in this field thus seems to be restricted to the observations that the presence of carbon dioxide is of great importance for the growth of the cells (ROCKWELL and HIGHERGER 1927), for the formation of succinic acid (KLEINZELLER 1941), and for the dehydrogenation reactions within the cells (HES 1938). Numerous investigations have shown, moreover, that the fermentation of glucose is not influenced by carbon dioxide under atmospheric pressure, while even minor concentrations of carbon dioxide in the gaseous phase affect the endogenous respiration in brewer's yeast (HAMON 1936).

A. Microscopical Observations.

Attempts were made by microscopical studies in visible light to find whether carbon dioxide had any visible effect on the protoplasm of the living cells. For this purpose, suspensions of baker's yeast were shaken in *M/15 Na-SS* buffer, pH 5.1, in an atmosphere containing carbon dioxide. The cytological picture of the cells thus treated then was compared with that in yeast simi-

larly shaken in air and in nitrogen. Neither after shaking the yeast for 24 hours at 25° C. in an atmosphere of air containing 25—50 % carbon dioxide nor after shaking in pure carbon dioxide could any change within the protoplasm, indicating a cell injury, be observed. In all cases, only the changes typical of starvation were observed in comparison with control experiments, *i. e.* weak vacuolization of the protoplasm and aggregation of the volutine grains, as described in the preceding chapter.

B. Effect on Growth.

The effect of carbon dioxide on the growth of yeast was investigated for different carbon dioxide concentrations in the gaseous phase and at various times, using different inoculation quantities of baker's yeast. The experiments were performed in 450 ml. culture flasks containing 30 ml. nutrient solution. The nutrient solution was inoculated with a KROGH syringe, through a tube in the stopper of the flask, containing 1 ml. yeast suspension in sterile water with a yeast content corresponding to 2.6 mg. or 5.2 mg. dry weight. Through a stopcock with a cotton filter, 2 litres of gas mixture, composed according to the data in Table 7, were run through the flasks. In order to correct for the decrease in oxygen pressure and the increase in carbon dioxide pressure during the experiment, the flasks were filled anew with 2 litres of the gaseous mixtures after 3 and 6 1/2 hours. At the end of the experiment, the yeast was sucked off, washed and dried. Table 7 gives the results obtained. The experiments performed at different carbon dioxide pressures and the corresponding controls were inoculated with different samples of manufactured baker's yeast. The values given in the table represent the means of two determinations.

The table shows an increased reduction in growth with increasing concentration of carbon dioxide. In the presence of 50 % carbon dioxide in the gaseous phase, the increase in weight after 10 hours was 33 % less than that of the control experiments. However, despite the high carbon dioxide concentration the yeast increased to ten times its original weight. This fact makes it obvious that the concentration of carbon dioxide in question cannot be regarded as injurious to the yeast. Further experiments which support this view may be found both in the following description and in Chapters IV and V.

Table 7.

Effect of CO_2 on growth of baker's yeast.

Temp.: 25° C.

mg. yeast injected (dry weight)	Time in hours	Initial gas mixture: 50 % O ₂	Increase		Reduction in growth in presence of CO ₂
			in mg. dry weight	in %	
2.6	10	+ 50 % N ₂	40.2	1545	10 %
	10	+ 15 % CO ₂ + 35 % N ₂	36.1	1390	
5.2	5	+ 50 % N ₂	12.9	248	8 %
	5	+ 15 % CO ₂ + 35 % N ₂	11.8	227	
2.6	10	+ 50 % N ₂	36.2	1390	12 %
	10	+ 25 % CO ₂ + 25 % N ₂	32.0	1230	
5.2	5	+ 50 % N ₂	11.7	225	7 %
	5	+ 25 % CO ₂ + 25 % N ₂	10.9	210	
2.6	10	+ 50 % N ₂	40.5	1555	33 %
	10	+ 50 % CO ₂	27.0	1040	
5.2	5	+ 50 % N ₂	14.2	273	23 %
	5	+ 50 % CO ₂	11.0	211	

According to investigations of the effect of carbon dioxide on the growth of fungi (see, *inter alia*, the works of PLATZ and co-workers and of GOLDING reviewed in Chapter I), the growth of yeast could not be expected to be already impeded at an initial concentration of 15 % carbon dioxide in the gaseous phase. It is, however, very probable that, if the experimental conditions were changed, no reduction in growth is obtained in the presence of the same carbon dioxide concentration. According to LOPRIORE and to HAMON, the pretreatment of the yeast strongly affects its reaction to carbon dioxide. As baker's yeast was cultivated under strong aeration, by which procedure the concentration of carbon dioxide in the gaseous phase was maintained at a low level, it was not adapted to the presence of moderate concentrations of carbon dioxide. It may thus be assumed that baker's yeast is relatively more sensitive to higher concentrations of carbon dioxide in the gaseous phase containing 50 % oxygen than yeast cultivated in not especially aerated laboratory cultures, where the concentration of carbon dioxide is frequently rather high.

Unpublished experiments on the effect of carbon dioxide on the growth of yeast were performed by NORMANDER at the Swedish Yeast Factory Co., on a semi-technical scale, using top yeast and cultivation by the method of differential fermentation. These

experiments showed that, in contrast to the experiments mentioned previously, no reduction was obtained after addition of 20 % carbon dioxide to the air blown through the suspension. On the other hand, it was observed that a mixture of 80 % carbon dioxide and 20 % oxygen strongly reduced the growth of yeast, simultaneously causing a greatly increased alcohol formation. The addition of carbon dioxide was interrupted after 8 hours and the aeration continued with oxygen alone. Subsequently, the growth of the yeast increased rapidly and the alcohol formation ceased, indicating that the retarding effect of carbon dioxide on the growth and the induction of an aerobic fermentation were reversible processes, despite the high concentration of carbon dioxide employed.

C. Effect on Endogenous Metabolism.

The endogenous metabolism of the yeast under aerobic conditions in the presence and absence of carbon dioxide in the gaseous phase was investigated by WARBURG's indirect method as described in the preceding chapter. The experiments were performed with gas mixtures of 20 % oxygen + 25 % carbon dioxide + 55 % nitrogen or 20 % oxygen + 50 % carbon dioxide + 30 % nitrogen. Measurements on controls with air in the gaseous phase were performed simultaneously. 100 mg. of baker's yeast was used in each apparatus. Tables 8 a and 8 b show the experimental results. The columns marked with the same number refer to one and the same experiment.

Tables 8 a and 8 b show that the endogenous respiration of baker's yeast in experiments both with 25 % and 50 % carbon dioxide is obviously greater than the corresponding metabolism in air during the time of the experiment (3 hours). In all cases, the value of the respiratory quotient is about 1, indicating that the metabolism activated by carbon dioxide originates in an increased break-down of carbohydrate. The increase in endogenous respiration of baker's yeast at increased carbon dioxide concentration agrees well with HAMON's (1936) observation that the presence of 5—30 % carbon dioxide in the gaseous phase activated the respiration of moist brewer's yeast, cultivated under aeration at 24° C.

Tables 8 a and b.

Effect of CO₂ on endogenous metabolism in baker's yeast.

The values are expressed in μl . per 20 min. per 100 mg. yeast.

a)

Exp. No.	Air								20 % O ₂ + 25 % CO ₂ + 55 % N ₂								
	O ₂ -consumption				CO ₂ -formation				O ₂ -consumption				CO ₂ -formation				
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean	
0—20 min.	69	75	70	71	60	71	65	65	97	131	75	101	91	128	71	97	
20—40	*	58	60	59	59	53	58	57	56	67	84	62	71	64	81	61	69
40—60	*	49	51	51	50	46	51	48	48	62	62	52	59	62	61	49	57
60—90	*	41	45	46	44	39	45	47	44	50	49	46	48	48	47	47	47
90—120	*	38	41	39	39	37	42	39	39	48	46	42	45	50	47	44	47
120—150	*	31	33	38	34	29	33	40	34	36	38	41	38	37	39	43	40
150—180	*	27	29	29	28	27	28	29	28	32	30	37	33	32	31	38	34
0—180	*	382	408	408	398	357	402	403	387	475	521	438	477	468	516	439	475

b)

Exp. No.	Air								20 % O ₂ + 50 % CO ₂ + 30 % N ₂								
	O ₂ -consumption				CO ₂ -formation				O ₂ -consumption				CO ₂ -formation				
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean	
0—20 min.	52	83	65	67	50	79	60	63	85	104	92	94	82	100	89	90	
20—40	*	46	59	51	52	43	56	47	49	74	81	83	79	76	82	83	80
40—60	*	39	49	44	44	38	46	41	42	64	62	47	58	66	63	47	59
60—90	*	37	43	41	40	37	42	40	40	52	48	43	48	54	50	42	49
90—120	*	34	39	29	34	35	39	27	34	48	38	36	41	51	37	42	42
120—150	*	27	32	26	28	28	32	26	29	40	35	38	36	42	36	35	38
150—180	*	28	25	—	27	29	25	—	27	38	33	—	36	41	35	—	38
0—180	*	326	399	—	357	325	388	—	349	490	478	—	473	506	482	—	480

D. Effect on Free Phosphate Content.

As indicated by the investigations of the phosphate fractions of yeast at different physiological conditions, described in the preceding chapter, the changes in the acid-soluble free phosphate content in the protoplasm of baker's yeast are sensitive indicators of the changes in the cell metabolism. The amount of free phosphate was therefore determined in yeast which, without addition of substrate, was shaken under aerobic conditions in an atmosphere with and without carbon dioxide. In order to determine whether carbon dioxide at the concentrations used caused a liberation of phosphate ions from the cells, the phosphate con-

tent of the suspension medium was also determined after shaking the yeast with and without carbon dioxide in the gaseous phase.

The experiments were performed in 450 ml. culture flasks to which were added 50 ml. suspension containing 4 g. of baker's yeast washed twice with $M/10$ Na-SS buffer, pH 5.1, and suspended in the same buffer. The suspensions were rapidly treated in a water thermostat (25° C.) for 10 minutes with a gas mixture of the composition given in Table 9. They were shaken subsequently with the gaseous phase for an additional 60 minutes. A part of each suspension was fixed with trichloroacetic acid for the determination of the free phosphate content of the cells; another part was cooled and centrifuged for determining the free phosphate in the suspension medium. Table 9 shows the effect of carbon dioxide under aerobic conditions on the free phosphate content of the cells and on the liberation of phosphate ions into the suspension medium.

Table 9.

Effect of CO_2 on free phosphate content in baker's yeast and on the liberation of phosphate into the suspension medium.

Composition of gaseous phase	Free phosphate in μ g. P per g. baker's yeast	
	In the cells	Liberated into the suspension medium
50 % O_2 + 50 % N_2	286	3
50 % O_2 + 50 % CO_2	229	1
50 % O_2 + 50 % N_2	284	2
50 % O_2 + 20 % CO_2 + 30 % N_2	253	1

As seen from the table, the amount of free phosphate in the protoplasm is decreased in the presence of carbon dioxide in the gaseous phase. Simultaneously with the decrease in free phosphate, a decrease in the amount of phosphate liberated into the suspension medium is observed. The errors in the determinations of such small amounts of phosphate are rather great. Nevertheless, in numerous experiments the free phosphate content of the suspension medium in the presence of carbon dioxide was always found to be lower than that obtained after shaking the yeast with oxygen and nitrogen.

Determinations of the free phosphate content of the cells were performed on baker's yeast and pretreated yeasts which were shaken under aerobic and anaerobic conditions without and with 25 % carbon dioxide in the gaseous phase. For a later estimation of factors possibly affecting the acidity of the cells it was of immediate interest to know the amount of free phosphate in the different yeasts after treatment analogous with that used in the determination of bound carbon dioxide in yeast (see Chapter IV). The suspensions of the different yeasts were therefore treated in trough-shaped WARBURG flasks containing 1.5 ml. suspension with 200 mg. of yeast in 0.059 *M* Na-SS buffer, pH 5.1. The apparatuses were filled with the gas mixtures shown in Table 10 while being shaken in the thermostat for 20 minutes. After further shaking for 17 minutes in the gas mixture, the contents of the flasks were fixed by addition of trichloroacetic acid. The amounts of free phosphate were estimated. The results may be seen from Table 10. The difference between the contents of free phosphate in a yeast treated with and without addition of carbon dioxide is expressed as a percentage of the value for the control in a CO₂-free atmosphere.

Table 10.

Effect of CO₂ on free phosphate content in baker's yeast and pretreated yeasts under aerobic and anaerobic conditions.

Composition of gaseous phase	Free phosphate in μ g. P per g. yeast				
	Baker's yeast		Fed yeast		Starved yeast
	Dif- fer- ence in %	Dif- fer- ence in %	Dif- fer- ence in %	Dif- fer- ence in %	Dif- fer- ence in %
50 % O ₂ + 50 % N ₂	261		156	155	298
50 % O ₂ + 25 % CO ₂ + 25 % N ₂	224	—14	145	144	291
N ₂	446	(—1)	231	189	404
25 % CO ₂ + 75 % N ₂	443		230	198	406

Just as in the preceding experiments, after treatment of baker's yeast under aerobic conditions in an atmosphere containing carbon dioxide, a smaller amount of free phosphate was found than in the control experiment. Both the fed and the budding yeasts showed a considerably lower free phosphate content than did baker's yeast, which may be associated with the much stronger

endogenous metabolism in pretreated yeasts as compared with baker's yeast.

The marked increase in free phosphate within the cells which was observed during shaking of the yeasts under anaerobic conditions is in good agreement with LYNEN's (1941) observations that the content of free phosphate in the yeast is higher in the anaerobic turnover of glucose than during aerobic metabolism. Table 10 demonstrates that the presence of carbon dioxide during aerobic treatment of the yeast obviously has a lowering effect on the free phosphate content of the protoplasm. The phenomenon appears less distinctly only in experiments on starved yeast. This agrees well, however, with the low capacity of starved yeast for binding carbon dioxide described in Chapter IV and with the relatively slight effect of this gas on the pH in the cells under aerobic conditions (Chapter V). Except in the case of budding yeast, carbon dioxide does not influence the phosphate content under anaerobic conditions, an observation which was confirmed by repeated experiments. The difference in the effect of carbon dioxide under aerobic and anaerobic conditions was also observed in investigations of the influence of the gas on numerous other reactions in yeast (cf. below).

Later descriptions of pH measurements and electrometric titrations on extracts and suspensions of yeast fixed by heat reveal that an extract from yeast shaken under aerobic conditions for 70 minutes in a gas mixture containing carbon dioxide has a remarkably higher pH value than an extract from yeast shaken in a mixture of nitrogen and oxygen alone. In an attempt to find the cause of this increase in pH, the content of free phosphate was investigated also in these extracts. 10 g. of baker's yeast was washed twice and suspended in 65 ml. distilled water. The suspension was shaken at 25° C. for 70 minutes in a 450 ml. culture flask containing 50 % oxygen + 25 % carbon dioxide + 25 % nitrogen. Controls were shaken simultaneously in 50 % oxygen + 50 % nitrogen. After the shaking the suspensions were cooled and centrifuged. The yeast was suspended in 3 ml. distilled water and was heat-fixed immediately, according to the description given in Chapter V. The heat-fixed suspensions were centrifuged and the clear extract was precipitated with trichloroacetic acid. Estimations from a series of experiments exhibited a free phosphate content corresponding to 0.40—0.45 mg. P per ml. extract from yeast treated without carbon dioxide. The free phos-

phate content in extracts from yeast shaken in the gaseous phase containing carbon dioxide was always 14—15 % lower, which is in agreement with the preceding experiments.

E. Effect on Ammonium Ion Content.

As was mentioned in Chapter I, FIFE and co-workers (1935, 1941) have shown that after treatment of different parts of sugar beet plants in an atmosphere containing 20—60 % carbon dioxide, a liberation of ammonium ions was observed, possibly originating from the hydrolysis of amide-nitrogen. In order to find whether corresponding reactions occurred in yeast, the ammonia-nitrogen content of the cells was determined after shaking the yeast for 70 minutes in gaseous phases of 50 % oxygen + 50 % nitrogen and 50 % oxygen + 50 % carbon dioxide. Sufficient material was provided by shaking 8 g. of yeast in 60 ml. 0.059 *M* Na-SS buffer, pH 5.1, in a 450 ml. culture flask. The suspension was fixed with trichloroacetic acid and neutralized with hydroxide. Distillation of the fixed suspension and the centrifuged extract after addition of borate buffer to pH 9.2 yielded similar values. The content of ammonia-nitrogen in baker's yeast and in yeast shaken under aerobic conditions without carbon dioxide was only 2—3 μ g. N per g. yeast. After treatment in the atmosphere containing carbon dioxide, 3—4 μ g. N per g. yeast was found. Repeated experiments on distilled extract brought to a pH above 10 by addition of sodium carbonate or hydroxide gave the same low value for the ammonia-nitrogen content. A liberation of ammonium ions during shaking of the yeast under aerobic conditions in an atmosphere containing carbon dioxide is practically absent within these cells, as seen from the experimental results. When the amount of ammonia-nitrogen was estimated in the extracts of heat-fixed yeast, no significant difference could be observed in the content of ammonia-nitrogen in yeasts shaken with 50 % oxygen + 50 % nitrogen and 50 % oxygen + 25 % carbon dioxide + 25 % nitrogen in the gaseous phase. In these extracts, the ammonia-nitrogen content was 8—10 μ g. N per ml. extract.

F. Effect on Uptake of Glucose.

The effect of carbon dioxide on the uptake of glucose by baker's yeast under aerobic conditions was investigated by shaking yeast suspensions at 25° C. in a gaseous phase containing 50 %

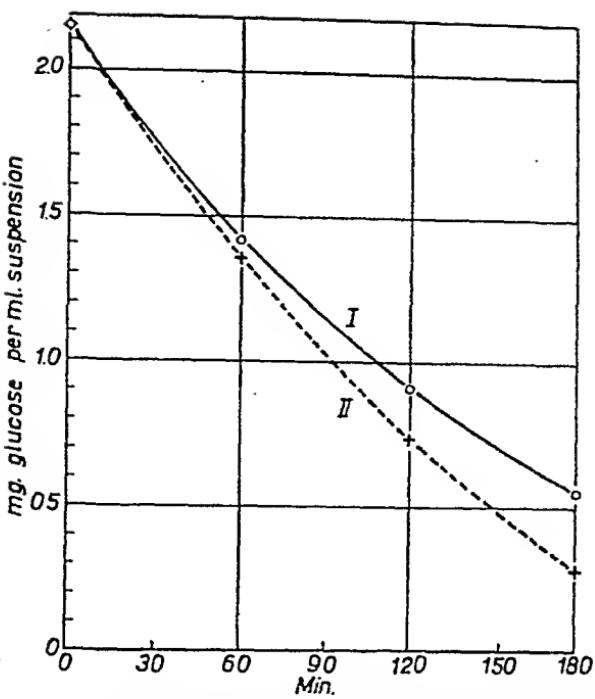


Fig. 17 a. Effect of CO_2 on the uptake of glucose by baker's yeast under aerobic conditions. Yeast concentration 0.83 %.

(I) Gaseous phase with 50 % $\text{O}_2 + 50\%$ N_2 .

(II) Gaseous phase with 50 % $\text{O}_2 + 15\%$ $\text{CO}_2 + 35\%$ N_2 .

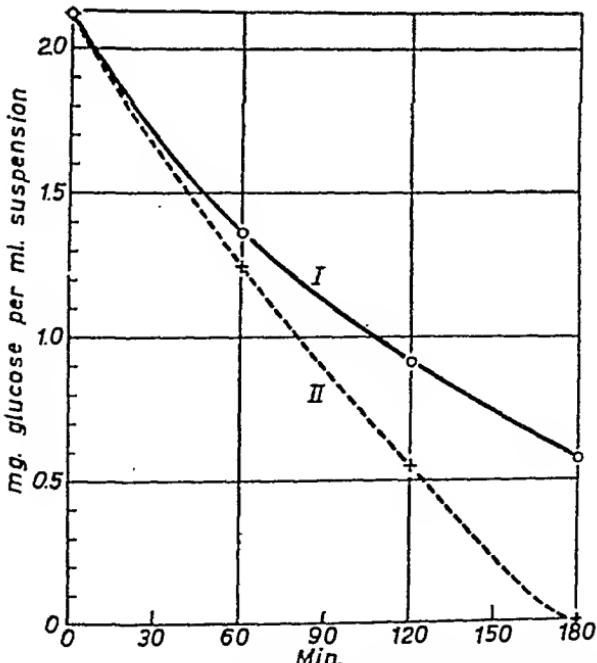


Fig. 17 b. Effect of CO_2 on the uptake of glucose by baker's yeast under aerobic conditions. Yeast concentration 0.83 %.

(I) Gaseous phase with 50 % $\text{O}_2 + 50\%$ N_2 .

(II) Gaseous phase with 50 % $\text{O}_2 + 50\%$ CO_2 .

oxygen and 0 %, 15 % or 50 % carbon dioxide. The experiments were performed in 450 ml. culture flasks containing 24 ml. of yeast suspension with 200 mg. of baker's yeast in 0.08 *M* Na-SS buffer, pH 5.1, and 50 mg. of glucose. The initial concentration of glucose was thus 2.1 mg. per ml. suspension. The amounts of glucose in samples of the suspensions were determined at intervals. The results are given in Figs. 17 a and 17 b.

The figures show that glucose is taken up more rapidly both in the presence of 15 % and 50 % carbon dioxide than in the presence of 50 % oxygen and 50 % nitrogen, as in the controls.

Corresponding experiments were carried out also with 50 % oxygen + 50 % nitrogen and 50 % oxygen + 25 % carbon dioxide + 25 % nitrogen in the presence of 4.5 mg. ammonium chloride per 24 ml. suspension. In view of the high rate at which glucose is taken up by yeast in the presence of ammonium chloride, the uptake of glucose was determined also in the presence of half the concentration of yeast, *viz.* 100 mg. baker's yeast per 24 ml. suspension. The results of these experiments are illustrated in Fig. 18.

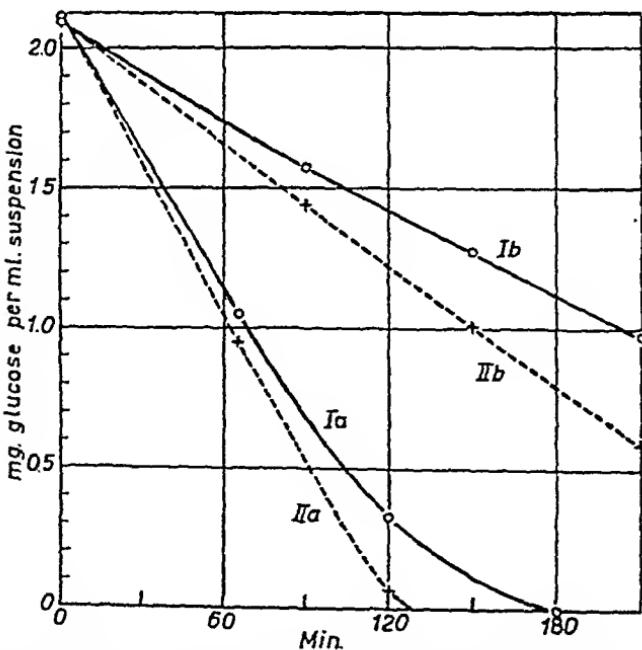


Fig. 18. Effect of CO_2 on the uptake of glucose by baker's yeast under aerobic conditions in the presence of NH_4Cl . Yeast concentration 0.83 % in I a and II a, 0.42 % in I b and II b.

- (I a) Gaseous phase with 50 % O_2 + 50 % N_2 .
- (II a) Gaseous phase with 50 % O_2 + 25 % CO_2 + 25 % N_2 .
- (I b) Gaseous phase with 50 % O_2 + 50 % N_2 .
- (II b) Gaseous phase with 50 % O_2 + 25 % CO_2 + 25 % N_2 .

As in the preceding experiment, carbon dioxide in the presence of ammonium chloride under aerobic conditions causes a more rapid uptake of glucose. A microscopical examination of the yeast showed that at the end of the experiment (210 min.) the cells of both suspensions had clearly started budding. Just as after the consumption of glucose alone, the content of the cells after the consumption of glucose in the presence of ammonium chloride showed in all cases a positive glycogen reaction after staining with LUGOL's solution.

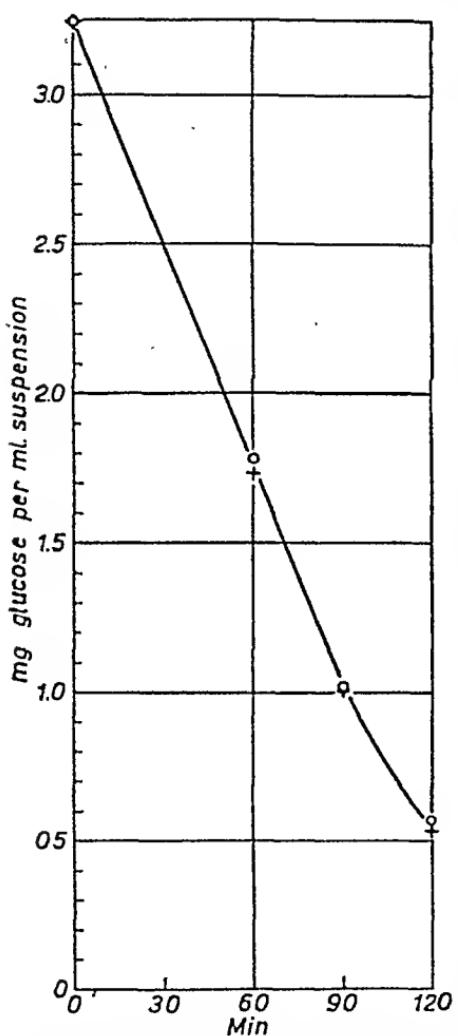


Fig. 19. Effect of CO_2 on the uptake of glucose by baker's yeast under anaerobic conditions. Yeast concentration 0.83 %.

- (o) Gaseous phase with N_2 .
- (+) Gaseous phase with 25 % CO_2 + 75 % N_2 .

Determinations of the uptake of glucose were also made under anaerobic conditions, employing trough-shaped WARBURG flasks containing 2.4 ml. suspension with a glucose concentration corresponding to 3.2 mg. per ml. suspension. The gaseous phase consisted either of pure nitrogen or 25 % carbon dioxide + 75 % nitrogen. Fig. 19 illustrates the uptake of glucose during shaking of the suspensions in the two atmospheres.

In contrast to the preceding experiments under aerobic conditions, no effect of carbon dioxide on the uptake of glucose could be detected under anaerobic conditions. Similar experiments were carried out with 2.1 mg. glucose per ml. suspension; they led to the same result.

For further confirmation of the absence of an effect by carbon dioxide on the anaerobic turnover of glucose, the fermentation carbon dioxide developed was measured in the WARBURG apparatus. In experiments performed in a nitrogen atmosphere, without and with 25 % carbon dioxide, complete agreement was always found between the curves for the carbon dioxide evolved.

of glucose, the fermentation carbon dioxide developed was measured in the WARBURG apparatus. In experiments performed in a nitrogen atmosphere, without and with 25 % carbon dioxide, complete agreement was always found between the curves for the carbon dioxide evolved.

G. Effect on Uptake of Pyruvic Acid.

The effect of carbon dioxide on the uptake of pyruvic acid by baker's yeast was finally investigated. Since pyruvic acid is consumed very slowly by baker's yeast under anaerobic conditions (cf. RUNNSTRÖM, SPERBER and KARLSSON 1939), the experiments were carried out exclusively under aerobic conditions. 450 ml. culture flasks contained 24 ml. of yeast suspension with 400 mg. of baker's yeast in 0.08 *M* Na-SS buffer, pH 5.1. In two experiments, the amounts of pyruvic acid added were 417 and 523 μ mol., respectively, per 24 ml. suspension. The contents were shaken in atmospheres without and with 15 % carbon dioxide. After periods of 90 and 180 minutes, the amounts of pyruvic acid present in the suspension media were estimated. Table 11 shows the result of these experiments.

Table 11.

Effect of CO₂ on uptake of pyruvic acid by baker's yeast.

Composition of gaseous phase	μ mol. pyruvic acid		
	Amount added	Amount taken up	
		after 90 min.	after 180 min.
50 % O ₂ + 50 % N ₂	417	100	191
50 % O ₂ + 15 % CO ₂ + 35 % N ₂	417	105	209
50 % O ₂ + 50 % N ₂	523	84	147
50 % O ₂ + 15 % CO ₂ + 35 % N ₂	523	88	165

The table shows that, when the gaseous phase contained 15 % carbon dioxide, a markedly larger amount of pyruvic acid was taken up than in the presence of oxygen-nitrogen. This agrees well with the observations made by SPERBER and RUNNSTRÖM (1939). In their experiments on the aerobic turnover of pyruvic acid by baker's yeast, these authors shook suspensions in WARBURG vessels with and without potassium hydroxide in the central tube. They found that the consumption of pyruvic acid was generally somewhat higher in the vessels in which carbon dioxide was not absorbed.

H. Summary of Chapter III.

From experiments on the effect of carbon dioxide on yeast *under aerobic conditions* it appears that the growth of baker's yeast is already reduced in the presence of 15 % carbon dioxide in the gaseous phase. The endogenous respiration of this yeast is, however, increased even when relatively high concentrations of carbon dioxide are present. Both in baker's yeast and in fed and budding yeasts obtained by pretreatment of baker's yeast the presence of 25 % carbon dioxide initiates a reduction in the free phosphate content of the cells. In experiments on baker's yeast, no liberation of phosphate ions into the suspension medium can be detected, which might have been caused by the presence of carbon dioxide. Nor does the presence of 50 % carbon dioxide cause any formation of ammonium ions in the protoplasm. The uptakes of glucose and pyruvic acid are more rapid under the influence of carbon dioxide. The effect of carbon dioxide on the aerobic metabolism of baker's yeast is also revealed by investigations discussed in the preceding chapter (Table 5) concerning the formation of alcohol during aerobic fermentation. In the presence of carbon dioxide an increased alcohol production is observed.

In contrast to the observations previously mentioned, carbon dioxide does not affect the metabolism and the cell constituents in yeast *under anaerobic conditions*. This appears from the fact that the free phosphate content in the yeast is independent of the concentration of carbon dioxide in the gaseous phase (except in the case of budding yeast). Moreover, the rates at which glucose is taken up and fermented by yeast under anaerobic conditions are not influenced by carbon dioxide.

The discrepancies observed between the effect of carbon dioxide under aerobic and anaerobic conditions are further elucidated by the observations described in the following chapters, which concern the ability of yeast to bind carbon dioxide and the effect of carbon dioxide on the acidity of the living cells.

CHAPTER IV.

Determination of Acid-labile Bound Carbon Dioxide in the Yeasts.

A. Introduction.

In the following account, carbon dioxide liberated by acidification from the content of cells is denoted as bound carbon dioxide. In the earlier literature, it was generally assumed that bound carbon dioxide largely originated in the decomposition of bicarbonate. The amount of carbon dioxide liberated from carbonate within the protoplasm can be completely neglected, since the cell content is generally more or less neutral, the content of carbonate in the protoplasm therefore being very small. Investigations performed during recent years on, *inter alia*, blood corpuscles, have shown, however, that the bound carbon dioxide is not only present as bicarbonate, but can also be reversibly bound as carbamate and possibly exists in another still unknown form (see Chapter VI). Whether the bound carbon dioxide in yeast also partly originates from other sources than bicarbonate will be discussed in Chapter VI. It may be emphasized here, however, that carbon dioxide bound in yeast cannot, without further consideration, be assumed to originate solely in bicarbonate.

Determinations of the amount of bound carbon dioxide in, for example, blood or muscle tissue are generally based upon gas analyses made with VAN SLYKE's apparatus. In this procedure, the material is acidified and carbon dioxide is removed by evacuation; it is subsequently collected and measured manometrically. (For different modifications of appropriate methods, cf. PETERS and VAN SLYKE 1932, and DANIELSON and HASTINGS 1939.) Instead of employing any of these methods, the present author determined the amount of carbon dioxide bound in yeast, using the principle of the WARBURG method. The amount of car-

bon dioxide expelled from the cells was determined manometrically by tilting a strong, non-volatile acid into a yeast suspension in equilibrium with a gaseous phase with a given carbon dioxide concentration. When the cells are rapidly fixed with acid, this method permits us to estimate the amount of bound carbon dioxide present in the yeast, despite the endogenous metabolism before acidification. This method, furthermore, makes it possible to determine the amount of bound carbon dioxide for a given physiological state of the cells, *e. g.* under aerobic or anaerobic conditions at any desired carbon dioxide pressure and at any time.

It is a necessary condition for the determination of the amount of bound carbon dioxide in yeast that the yeast is fixed immediately after addition of the acid. Consequently, a special investigation was performed with the aim of finding a suitable acid for this purpose. Furthermore, the calculations of the vessel constants and the checking of blanks require knowledge of the solubility of carbon dioxide in the acid, the SS buffer and the mixture obtained after tilting. The necessary values of the absorption coefficients for carbon dioxide were determined. In order to elaborate the final method, various determinations were indispensable, *viz.* the determination of the appropriate amount of yeast, the rate of shaking, and the times of gas passage and shaking before the tipping of the acid. A special procedure was developed for obtaining reliable blank values.

B. Method.

1. Development of the Method.

In the manometric method, trough-shaped WARBURG vessels with plane bottom, one side-bulb with a gas outlet stopper, and without central tube were employed. The volume of each vessel was 15—17 ml. The main compartment contained 1.50 ml. yeast suspension in SS buffer; the bulb contained 0.30 ml. acid. The rate of shaking was 100 complete oscillations per minute at an amplitude of 4 cm. In all experiments the temperature was 25° C. The WARBURG flasks were filled with gas during shaking in the thermostat, whereby 4 groups, each consisting of 3 flasks connected in series with rubber tubes, were treated with one litre of gas mixture per group. The time of gas passage was 20—25 minutes, after which period complete gas equilibrium had been reached. In all cases, gasometers were used for the treatment (cf. p. 36).

a) Choice of Acid for the Fixation of Yeast and the Expulsion of Carbon Dioxide.

Owing to the endogenous respiration and fermentation, a continuous change in the gas pressure prevails in the WARBURG vessels. In the manometric determination of carbon dioxide, this pressure change must be stopped immediately by a rapid fixation of the yeast with acid. The acid employed should thus have a rapid fixing action and should not be volatile, since amounts might otherwise distil from the bulb, before the tipping, into the yeast suspension in the main compartment, and the manometric determination of the pressure might be disturbed.

In order to determine the rate of fixation, it was investigated whether the gas pressure became constant immediately after addition of acid to the yeast suspension. In these determinations, each flask was supplied with 1.50 ml. suspension containing 100 mg. baker's yeast washed twice with distilled water and suspended

Table 12.
Effect of various acids on baker's yeast.

Acid	Concen- tra-tion after tilting	Time after which an- aerobic CO ₂ - formation begins to fall off (in min.)	Time elaps- ing before plasma is granular in most cells (in min.)	Remarks
Sulphuric acid.	N/3	> 185	> 120	
" "	N	c. 70	c. 60	
Metaphosphoric acid	2/3 M	> 135	> 100	Under aerobic conditions during the first 10 min. neg. pressure
Orthophospho- ric acid	M/2	> 165	> 120	
Glyceric acid ..	2/3 M	c. 60	> 60	Pressure increment in air greater than in N ₂
Trichloroacetic acid	M/3	(< 10)	< 2	
Monochloroac- tic acid	2/3 M	(< 10)	< 2	Pressure disturbed by vap- our pressure of acids, this perhaps affecting yeast metabolism
Trichlorolactic acid	M/2	(< 10)	< 2	
Sulphosalicylic acid	M/3	< 10	c. 3	
Perchloric acid.	M/2	< 10	c. 2	
p-toluenesul- phonic acid..	0.61 M	< 10	< 2	

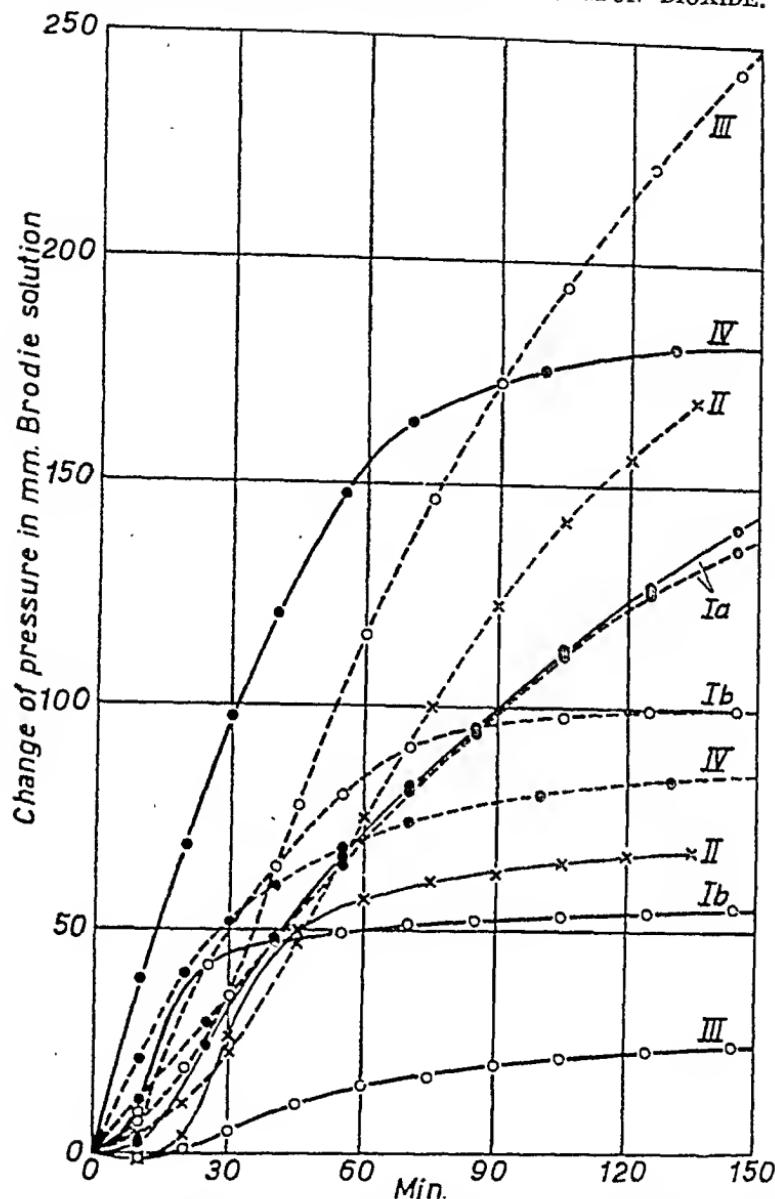


Fig. 20. Changes in pressure in manometric determinations of the effect of different acids on baker's yeast. Volume of vessel c. 15 ml. 100 mg. yeast per 1.8 ml. mixture of yeast suspension and acid.

— under aerobic conditions.

- - - under anaerobic conditions.

(I a)	After addition of H_2SO_4 ;	concentration after tilting N/3.
(I b)	» » » H_2SO_4 ;	» » » N.
(II)	» » » HPO_4 ;	» » » $2/3 M$.
(III)	» » » H_2PO_4 ;	» » » $M/2$.
(IV)	» » » glyceric acid;	» » » $2/3 M$.

in 0.063 *M* Na-SS buffer, pH 5.1. The bulb contained 0.30 ml. acid.

When seeking an acid suitable for the liberation of carbon dioxide, it was found that most acids did not penetrate and fix the yeast cells rapidly enough. Experiments performed with a gaseous phase containing air or nitrogen showed that, during the time of fixation of the cells, continuous increases in pressure were obtained (carbon dioxide production), the duration of which agreed well with microscopical observations of the time elapsing before the protoplasm was coagulated by the acid. Examples are given in Table 12 and Fig. 20.

It results from Table 12 and the curves that, when moderate concentrations of sulphuric acid, ortho- and metaphosphoric acids and glyceric acid were used, constant pressure was not obtained after the first reading, *i. e.* 10 minutes after tipping the acid. The continuous change in pressure under the influence of these acids could be almost completely inhibited by the addition of different protein-precipitating substances. A mixture of monochloro- and monoiodo-acetic acids with sulphuric acid completely removed the pressure change after the first reading period, as the concentration of these acetic acids after tipping was 0.017 *N* in 0.83 *N* sulphuric acid. When the halogen-substituted aliphatic acids were used alone, no satisfactorily constant pressure was obtained after tipping, despite a presumably very rapid fixation of the cells.

The experiments were completed by determining the respiration and carbon dioxide production obtained after tipping sulphuric acid of different concentrations into the yeast suspension. In these experiments, two-bulbed conical vessels with a central tube for potassium hydroxide or water were employed. The experiments were performed on 50 mg. baker's yeast per apparatus. The volumes of suspension and acid were similar to those in the preceding experiments.

The curves in Fig. 21 show that, immediately after the tipping of the sulphuric acid, increased endogenous metabolism with clearly aerobic fermentation occurs. Under the influence of *N/6* sulphuric acid, strong respiration and carbon dioxide formation are obtained during the whole period of the experiment. Not even in *N* sulphuric acid is the metabolism of the cells completely inhibited in the first minutes; respiration and carbon dioxide production do not cease until after about 30 minutes.

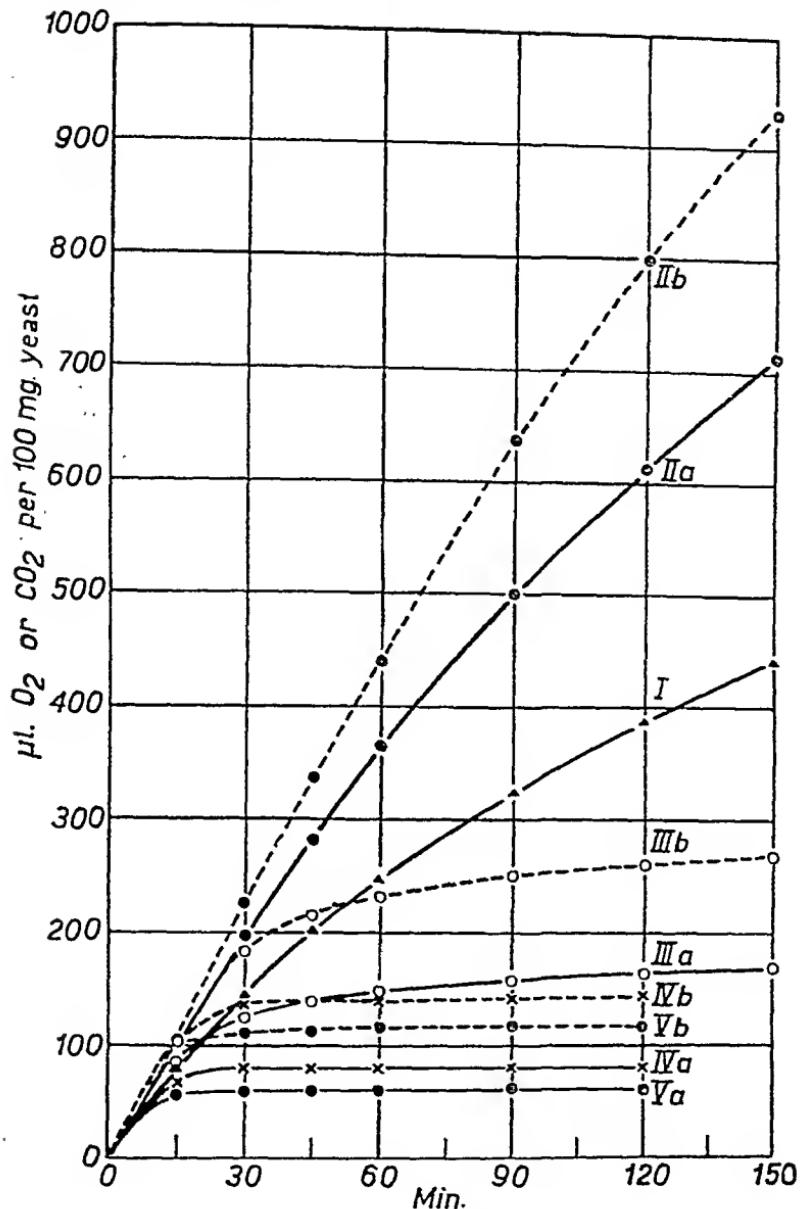


Fig. 21. Effect of different concentrations of H_2SO_4 on the endogenous metabolism of yeast under aerobic conditions.

— O₂-consumption. - - - CO₂-formation.

(I) Without addition of acid (respiratory quotient = 1).

(II) Concentration of H_2SO_4 after tilting $\frac{N}{6}$.

(III) \triangleright \triangleright \triangleright \triangleright \triangleright $\frac{N}{3}$.

(IV) \triangleright \triangleright \triangleright \triangleright \triangleright $\frac{2}{3} N$.

(V) \triangleright \triangleright \triangleright \triangleright \triangleright N .

The penetration of the acids examined is possibly counteracted to a large extent by the coagulate which, owing to the protein-precipitating qualities of the acids, is formed in the cell surface. This may explain why metaphosphoric acid is unsuitable for the fixation of yeast. More or less "lipid-soluble" acids are best suited. When the effect of p-toluenesulphonic acid in the final concentration 0.61 M is compared with those of $\frac{2}{3}\text{ M}$ monochloroacetic acid and N perchloric acid, all values for bound carbon dioxide are approximately equal in experiments performed with the same yeast in an atmosphere of $25\% \text{ CO}_2 + 75\% \text{ air}$. (For details of the method, see below. The values are calculated by subtracting the blank values obtained when the corresponding acids are tipped into $M/15$ SS buffer alone.) In view of the danger that perchloric acid might later prove to react with the yeast and thus initiate possible sources of error, this acid has not been used in the following experiments.

Among the acids investigated, p-toluenesulphonic acid proved to be most suited, being a strong, non-volatile, protein-precipitating and "lipid-soluble" acid. In the following experiments, p-toluenesulphonic acid was used exclusively. Before tipping, its concentration was 3.64 M (c. 68.9 g. p-toluenesulphonic acid (SCHERING-KAHLBAUM) diluted with water to 100 ml. The specific gravity of the solution was 1.18 at 20° C . The variation in values obtained with different solutions amounted to about 0.5 %.) When a calibrated 1 ml. pipette was used for taking the sample, and the acid was titrated with sodium hydroxide (indicator: bromocresol green), the values obtained were about 3.0—3.5 % too low, owing to the viscosity of the solution causing the acid to adhere to the pipette-wall. Samples taken with a KROGH syringe (0.299 ml.), however, yielded values agreeing well with those from weighed samples.

After the tipping of p-toluenesulphonic acid, the pressure in the apparatuses was stabilized rapidly. During the experiment, readings were performed every third minute. When 3.64 M p-toluenesulphonic acid was added to the yeast suspension shaken in air, in nitrogen or in a gas mixture with $50\% \text{ O}_2 + 25\% \text{ CO}_2 + 25\% \text{ N}_2$, the pressure in the flasks was already constant after 3 minutes. On the other hand, when 1.8 M acid was employed, the pressure only became constant after 6 minutes.

b) Determination of the Solubility of Carbon Dioxide in the Suspension Medium.

In the determination of the amount of bound carbon dioxide within the yeast cells in an atmosphere containing carbon dioxide, control experiments showed that when 0.30 ml. 3.64 M p-toluenesulphonic acid was tipped into 1.50 ml. $M/15$ Na-SS buffer, pH 5.1, large positive manometric deflections were obtained. These were presumably conditioned by a higher solubility of carbon dioxide in either the acid or the buffer than that in a mixture of these two liquids. The solubility of carbon dioxide was determined both in the buffer, in p-toluenesulphonic acid, and in mixtures of these solutions, and the above supposition was confirmed. A reliable value for the solubility of carbon dioxide in a mixture of buffer and acid is necessary for an accurate estimation of the vessel constants after the tipping of the acid.

The solubility of carbon dioxide was expressed in the form of BUNSEN's absorption coefficient (α). The values obtained were determined at 25° C. and give the amount of carbon dioxide in ml. (reduced to 760 mm. Hg and 0° C.), dissolved in 1 ml. solvent at 25° C. Since the pH of the buffer is 5, a certain amount of carbon dioxide originating from bicarbonate is included in the value of α for the buffer. This quantity is, however, very small at the pH in question.

The carbon dioxide was determined gravimetrically after absorption of the gas in "ascarite". The solution, the absorption coefficient of which was to be estimated, was saturated with chemically pure carbon dioxide (cf. Chapter II, A) in a test-tube. This tube was closed with a stopper provided with an inlet and outlet for the gas and a hole for the tip of a KROGH syringe of 7.12 ml. capacity. The tip of the syringe extended into the liquid in the tube. The solution was saturated with gas at a known atmospheric pressure in the thermostat at 25° C. by passing 1.5—2 l. of carbon dioxide from the gasometer for an hour at about 40 mm. water over-pressure (initial pressure for the bubbles). During the saturation, the syringe was filled with solution and cautiously emptied several times. After gas equilibrium was reached, the syringe was filled with solution and was immediately fixed in a test-tube by means of a tight rubber stopper. This test-tube was previously coupled into the apparatus for the CO₂ determination (Fig. 22).

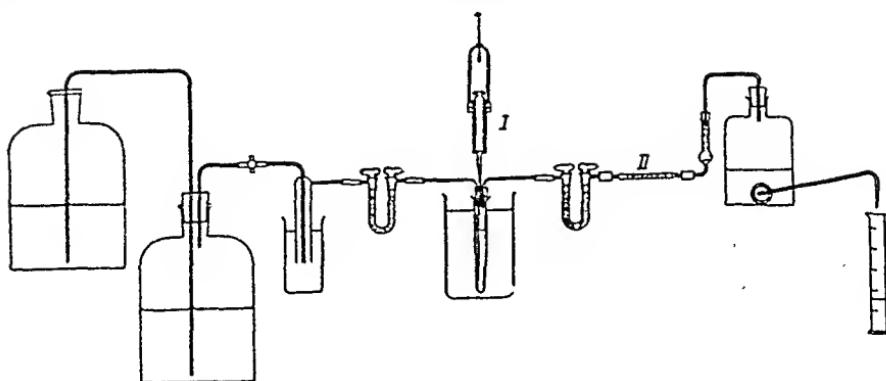


Fig. 22. Apparatus for the determination of the absorption coefficient of CO_2 . Sample of the solution in equilibrium with pure CO_2 was taken with a KROGH syringe (I) subsequently connected to the apparatus. The expelled gas was absorbed by "ascarite" in a PREGL tube (II).

Before the insertion of the syringe, 0.50 ml. 2 N sulphuric acid had been pipetted into the test-tube. The U-tube placed before the test-tube contained soda-asbestos in order to remove traces of carbon dioxide from the air passed through the sample. The subsequent U-tube was filled with CO_2 -treated coarse and fine grained calcium chloride for the drying of the gas containing the carbon dioxide. At II, a PREGL micro-absorption tube was inserted, containing "ascarite" and magnesium persulphate and a safety-tube containing calcium chloride. The remaining parts of the apparatus served to regulate the pressure and the gas flow. All joints were treated with paraffin oil *in vacuo* at 60° C. In order to expel the carbon dioxide from the test solution, the syringe was slowly emptied while a weak air stream was passed through the contents of the test-tube. After 50 ml. air had passed, the test-tube was warmed in a water bath to 40° C. and 200 ml. air were led through at this temperature over about 40 minutes. The carbon dioxide expelled was absorbed in the micro-absorption tube and weighed on the micro-balance.

α was determined in Na-SS buffer, pH 5, at different dilutions, in 3.64 M and 0.61 M p-toluenesulphonic acid, and in a mixture of 1 volume 3.64 M p-toluenesulphonic acid and 5 volumes M/15 Na-SS buffer. In the calculations of α , the value 22.26 l. was taken for the molar volume of carbon dioxide at 760 mm. Hg and 0° C. (GUYE and PINTZA 1908). In all cases, the volume of the sample was 7.12 ml. The weight of carbon dioxide obtained was recalculated for treatment with a carbon dioxide pressure of 760 mm. Hg. The α -values obtained in this manner include errors arising

from the vapour pressures of the various solutions and possible over-pressure during treatment of the solutions with carbon dioxide. Corrections for these factors were not made. For an estimation of the correctness of the values, the method was tested by determining α in water, in $N/2$ sulphuric acid, and in $M/2$ potassium chloride and comparing the values obtained with those given in the literature. The present determinations were found to be about 0.5 % too low as compared with the known values; all values determined were therefore raised by 0.5 % in order to obtain probable values for α . The mean value for the standard deviation σ was found to be 0.0028. This value was calculated from the pooled variances in all determinations (see FISHER 1944), as there was no reason to suppose different variabilities in the different solutions.

Table 13 shows the value of α determined according to the method described above together with corresponding values given in the literature.

Table 13.
Absorption coefficients of CO_2 .

Solution	CO ₂ absorbed per sample in mg. (calculated for equilibrium at 760 mm. Hg pressure of CO ₂)	$\alpha_{CO_2}^{25^\circ}$			Deviation from values given in literature
		Found	Mean	Literature	
H ₂ O	10.63	0.755	0.755±0.0009	(MARKHAM and KOBE 1941)	—0.2 %
	10.62	0.755			
	10.68	0.759			
	10.61	0.754			
	10.62	0.755			
N/2 H ₂ SO ₄	10.17	0.723	0.721±0.0014	0.727 (GEFFCKEN 1904)	—0.8 %
	10.09	0.717			
	10.15	0.721			
	10.17	0.723			
M/2 KCl	9.73	0.691	0.691±0.0009	0.6937 (MARKHAM and KOBE 1941)	—0.4 %
	9.74	0.692			
	9.70	0.689			

Table 14 gives the values obtained for α in different dilutions of SS-buffer, in p-toluenesulphonic acid, and in a mixture of buffer and acid.

Table 14.

Absorption coefficients of CO_2 in Na-SS buffer, p-toluenesulphonic acid and in a mixture of buffer and acid.

Solution	CO ₂ absorbed per sample in mg. (calculated for equilibrium at 760 mm. Hg pressure of CO ₂)	$\alpha_{\text{CO}_2}^{25^\circ}$		
		Found	Uncorrected mean	Corrected mean (Correction: + 0.004)
<i>M/5</i> Na-SS buffer, pH 5	10.46	0.743	0.743 ± 0.0004	0.747
	10.45	0.742		
	10.46	0.743		
0.083 <i>M</i> Na-SS buffer, pH 5	10.99	0.781	0.782 ± 0.0012	0.786
	10.98	0.780		
	11.04	0.784		
<i>M/15</i> Na-SS buffer, pH 5	11.07	0.786	0.784 ± 0.0015	0.788
	10.97	0.779		
	11.09	0.788		
	11.02	0.783		
	11.05	0.785		
0.046 <i>M</i> Na-SS buffer, pH 5	11.04	0.784	0.790 ± 0.0019	0.794
	11.15	0.792		
	11.15	0.792		
	11.13	0.791		
<i>M/50</i> Na-SS buffer, pH 5	11.14	0.791	0.792 ± 0.0006	0.796
	11.16	0.793		
	11.14	0.792		
3.64 <i>M</i> p-toluene-sulphonie acid	10.92	0.776	0.772 ± 0.0027	0.776
	10.90	0.774		
	10.80	0.767		
0.61 <i>M</i> p-toluene-sulphonic acid	10.39	0.738	0.735 ± 0.0021	0.739
	10.29	0.731		
	10.35	0.736		
1 vol. 3.64 <i>M</i> p-toluenesulphonic acid + 5 vol. <i>M/15</i> Na-SS buffer	10.17	0.723	0.724 ± 0.0009	0.728
	10.22	0.726		
	10.20	0.725		
	10.17	0.722		

Fig. 23 shows the variation of α_{CO_2} with the dilution of the buffer. This variation does not originate in an increased bicarbonate content, which might result from an increase in pH during dilution of the buffer. It has been shown previously (cf. Fig. 4) that the pH of the buffer increases during dilution in equilibrium with air; measurements of the pH of the buffer in equilibrium with carbon dioxide in the gaseous phase, however, have shown

that, in consequence of the reduction in buffering capacity with increased dilution, the pH, as compared to the pH in air, decreases more strongly in the more dilute buffer solutions. The increase in pH with dilution is thus compensated and the pH values of solutions in a gaseous phase containing carbon dioxide remain practically constant. The pH is 5.06 for $M/50$ Na-SS buffer, and 5.10 for $M/15$ buffer, in equilibrium with pure carbon dioxide at 19° C.

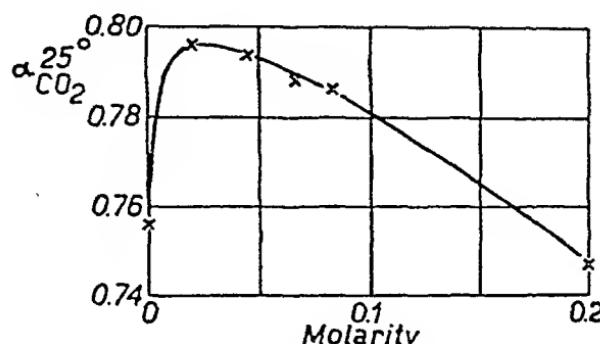


Fig. 23. Absorption coefficient for CO_2 in Na-SS buffer, pH 5, at different buffer concentrations.

c) Definitive Experimental Procedure.

In manometric estimations of the amount of acid-labile bound carbon dioxide in yeast, the WARBURG vessel contained 1.50 ml. suspension with, when not otherwise stated, 200 mg. of baker's yeast or the corresponding quantity of another yeast in 0.059 M Na-SS buffer, pH 5.1. The bulb contained 0.30 ml. 3.64 M p-toluenesulphonic acid. For the calculation of the vessel constants after the tipping of the acid, the above-determined value for $\alpha_{CO_2}^{25^\circ}$, 0.728, was used for the mixture of 1 volume 3.64 M p-toluenesulphonic acid + 5 vol. $M/15$ buffer. Owing to the dilution with intercellular water from the yeast, the buffer concentration in the presence of yeast was lower than $M/15$, which concentration it was originally intended to employ. Errors in the calculation of the vessel constants due to the use of the value for α in such mixtures may be neglected in experiments with 0.059 M buffer. Each determination was made in triplicate.

The experiments were carried out with a gaseous phase containing air, nitrogen or a mixture of nitrogen and oxygen, and also in gas mixtures, where part of the nitrogen was replaced by

carbon dioxide. In order to ensure a uniform treatment of the material, air, nitrogen or gas mixture was passed simultaneously through all 12 flasks during shaking in the thermostat (25° C) for 23 minutes. In the experiments in air, aeration was performed in order to remove the main quantity of carbon dioxide developed during endogenous respiration of the yeast. This is of importance, since the amount of bound carbon dioxide in the yeast in experiments without carbon dioxide is markedly affected even by a low carbon dioxide pressure.

As will be shown below, the amount of carbon dioxide expelled from the cells is dependent upon the endogenous metabolism of the yeast, which decreases with time. The period from the preparation of the solutions to the tipping of the acid was always kept as close as possible to 55 minutes. After preparing the suspension, about 11 minutes were spent in filling the apparatuses, placing them in the thermostat, and preparing the passage of gas. Then 5 minutes were devoted to preliminary shaking, 23 minutes to the passage of gas, 6 minutes to shaking for the attainment of complete equilibrium before the first reading. Another 10 minutes passed before the second reading was performed, the pressure changes being checked in the apparatuses immediately before the acid was tipped. The times were so lagged that the experiments of the first series of 6 apparatuses could be started two minutes before those of the second series. The pressure changes were read 10 minutes after tipping, and the constancy of the pressure was checked after a further 10 minutes.

2. Experiments with Gaseous Phase Free from Carbon Dioxide.

Blanks.

When p-toluenesulphonic acid was tipped into $M/15$ buffer, no significant change in the pressure was observed if the experiments were performed in gaseous phases containing air, nitrogen or a mixture of oxygen and nitrogen. No changes in the solubility of oxygen or nitrogen in the acid and the buffer before and after mixing, nor any contraction or dilatation of the acid during dilution with the buffer, factors which might affect the manometer readings, could be observed (see also Table 16).

Experiments with Yeast.

Table 15 gives the mean values for the amounts of carbon dioxide found in experiments with baker's yeast and the pretreated yeasts described in Chapter II, B.

Table 15.

*Bound CO₂ in baker's yeast and in pretreated yeasts.
Gaseous phase free from CO₂.*

Composition of gaseous phase	μl. CO ₂ per g. yeast				
	Baker's yeast	Starved yeast	Fed yeast	Budding yeast	Dried yeast (unwashed)
50 % O ₂ + 50 % N ₂	40	20	60—80	100—150	20
20 % O ₂ + 80 % N ₂	45	15	—	—	20
10 % O ₂ + 90 % N ₂	40	—	—	—	—
5 % O ₂ + 95 % N ₂	30	—	—	—	—
2 % O ₂ + 98 % N ₂	25	—	—	—	—
100 % N ₂	20	15	40	50	35

The amount of carbon dioxide expelled from the cells varies greatly in experiments with fed and budding yeasts under aerobic conditions. The experiments reveal that the amount of bound carbon dioxide is rather small, as the gaseous phase is practically free from carbon dioxide. It appears clearly from the table that, apart from the case of dried yeast, less carbon dioxide was bound in anaerobic than in aerobic experiments, a result which was confirmed by all subsequent experiments.

3. Preliminary Experiments in the Presence of Carbon Dioxide.

Blanks.

When the apparatuses are filled with gas mixtures containing carbon dioxide, an increase in the gas pressure is obtained after tipping of 0.30 ml. p-toluenesulphonic acid to 1.50 ml. M/15 Na-SS buffer, pH 5.1, as the solubility of carbon dioxide in the mixture is lower than in the acid and in the buffer. The amounts of carbon dioxide expelled from the mixture due to changes in α are seen in Table 16.

No significant difference was observed between the values

Table 16.

Blanks. $\mu\text{l. } \text{CO}_2$ expelled after mixing of 0.30 ml. *p*-toluenesulphonic acid and 1.50 ml. *M/15 Na-SS* buffer.

Composition of gaseous phase	$\mu\text{l. CO}_2$ after tilting	Number of determinations
50 % CO_2 + 50 % air	55.1 \pm 0.5	16
50 % CO_2 + 50 % O_2	55.8 \pm 0.5	11
50 % CO_2 + 50 % N_2	55.3 \pm 0.5	9
25 % CO_2 + 75 % air	28.6 \pm 0.3	10
25 % CO_2 + 75 % N_2	27.8 \pm 0.4	4
5 % CO_2 + 95 % air	5.8 \pm 0.3	6

obtained in experiments with the gaseous phase containing mixtures of carbon dioxide with air, oxygen or nitrogen in different concentrations. The amount of carbon dioxide given off is proportional to the concentration of carbon dioxide in the gaseous phase. In the present experiments, the variations in the air pressure were not considered, since their effect lay within the limits of accuracy of the method. Measurements performed at an air pressure 15 mm. Hg above or below the normal pressure might change the experimental values for 50 % carbon dioxide in the gaseous phase by about 1 μl . When calculations were made of the amounts of carbon dioxide liberated through changes in α after tipping, employing the values for α determined for the buffer, the acid, and a mixture thereof, in equilibrium with a gaseous phase containing 50 % carbon dioxide, the following values were obtained:

$$\frac{1}{2}(1,500 \cdot 0.788 + 300 \cdot 0.776 - 1,800 \cdot 0.728) = 52.2 \mu\text{l. CO}_2 \\ \text{calculated for } 0^\circ \text{ C. and 760 mm. Hg.}$$

These calculated values are in good agreement with the values found. Only a small variation in the values of α is necessary to cause a deviation in the calculated amounts of carbon dioxide of several $\mu\text{l.}$ from the original value.

The amounts of carbon dioxide evolved in the presence of yeast, in experiments with atmospheres containing carbon dioxide, proved to be too high in view of the carbon dioxide development in the blanks and had therefore to be corrected for the blank values. In the experiments with yeast in a gaseous phase with various contents of carbon dioxide, the blank value of 55 $\mu\text{l. CO}_2$ obtained from determinations in an atmosphere with 50 %

carbon dioxide was used as a preliminary basis for the correction of the carbon dioxide values measured. As will be shown below, however, the blank value is somewhat greater in the presence of yeast.

In order to check whether moderate variations in the concentration of p-toluenesulphonic acid had a noticeable effect on the blank value, experiments with three different concentrations of acid were performed. Table 17 shows the amounts of carbon dioxide given off from the solutions after the tipping of 0.30 ml. p-toluenesulphonic acid into 1.50 ml. $M/15$ Na-SS buffer, pH 5.1 (means of 4 determinations). The gaseous phase contained 50 % carbon dioxide and 50 % air.

Table 17.

Effect of variations in the p-toluenesulphonic acid concentration on the blank value.

p-toluenesulphonic acid		Blank value $\mu\text{l. CO}_2$
Concentration before tilting	Relative concentration	
3.53 M	97.0	52
3.64 M	100	55
3.84 M	105.5	58

A change of 1 % in the concentration of p-toluenesulphonic acid causes an error in the blank value of 1 $\mu\text{l. CO}_2$ at the most, despite the use of as high a concentration of carbon dioxide as 50 % in the gaseous phase. Since the variation in the concentration of p-toluenesulphonic acid in subsequent experiments was no more than about 0.5 %, this error may be neglected.

Experiments with Yeast.

Tables 18 a and b show, as typical examples, the results of two experiments in which the amount of bound carbon dioxide in baker's yeast was estimated. One of them (a) was carried out in a gaseous phase with constant oxygen concentration and varying carbon dioxide concentration, the other (b) with constant carbon dioxide concentration and varying oxygen concentration.

It results from Table 18 a that in experiments with the gaseous phase containing carbon dioxide and a high oxygen concentration,

Table 18.

Bound CO₂ in baker's yeast.

Yeast suspension: 4 g. baker's yeast, washed three times, + 16 ml. *M/10 Na-SS buffer*, pH 5.1, + 10 ml. distilled water.

1.50 ml. yeast suspension containing 200 mg. yeast + 0.30 ml. p-toluenesulphonic acid per sample. Different yeast samples were employed in experiments a and b.

a)

Composition of gaseous phase	$\mu\text{l. CO}_2$ after tilting		Blank value $\mu\text{l. CO}_2$ (uneorrected)	$\mu\text{l. CO}_2$ per 200 mg. yeast *)
	Single determination	Mean		
50 % O ₂ + 50 % N ₂	8 8 9	8	0	8
50 % O ₂ + 10 % CO ₂ + 40 % N ₂	48 47 49	48	11	37
50 % O ₂ + 25 % CO ₂ + 25 % N ₂	99 99 99	99	28	71
50 % O ₂ + 50 % CO ₂	162 162 164	163	55	108

b)

50 % CO ₂ + 1 % O ₂ + 49 % N ₂	81 80 78	80	55	25
50 % CO ₂ + 15 % O ₂ + 35 % N ₂	147 147 143	146	55	91
50 % CO ₂ + 25 % O ₂ + 25 % N ₂	164 160 162	162	55	107
50 % CO ₂ + 50 % O ₂	167 171 168	169	55	114

*) After preliminary correction.

a strong binding of carbon dioxide occurs, which increases with increasing carbon dioxide concentration. In a gaseous phase containing 50 % carbon dioxide, the yeast is not yet "saturated" with that gas.

The experiments summarized in Table 18 b were performed with a gaseous phase having the relatively high carbon dioxide content of 50 %. Just as in the experiments in air and in nitrogen, the amount of bound carbon dioxide in the cells is highly dependent on the oxygen concentration in the gaseous phase. The amount of carbon dioxide is reduced with decreasing oxygen concentration.

Table 19.

Bound CO₂ in starved yeast.

1.50 ml. yeast suspension containing 200 mg. starved yeast + 0.30 ml. p-toluene-sulphonic acid per sample. Different yeast samples were employed in experiments a and b.

a)

Composition of gaseous phase	$\mu\text{l. CO}_2$ after tilting		Blank value $\mu\text{l. CO}_2$ (uncorrected)	$\mu\text{l. CO}_2$ per 200 mg. yeast *)
	Single determina- tion	Mean		
50 % O ₂ + 50 % N ₂	3 5 4	4	0	4
50 % O ₂ + 10 % CO ₂ + 40 % N ₂	19 20 20	20	11	9
50 % O ₂ + 25 % CO ₂ + 25 % N ₂	44 43 46	44	28	16
50 % O ₂ + 50 % CO ₂	83 85 82	83	55	28

b)

50 % CO ₂ + 50 % N ₂	67 66 68	67	55	12
50 % CO ₂ + 1 % O ₂ + 49 % N ₂	71 72 70	71	55	16
50 % CO ₂ + 25 % O ₂ + 25 % N ₂	81 — 84	83	55	28
50 % CO ₂ + 50 % O ₂	82 86 87	85	55	30

*) After preliminary correction.

In order to obtain the maximum amount of bound carbon dioxide at the carbon dioxide concentration in question, the oxygen content of air is insufficient under the experimental conditions prevailing. Not even with a gaseous phase containing 25 % oxygen is the optimum amount of carbon dioxide bound. In all experiments similar to that in Table 18 a, an atmosphere containing 50 % oxygen was used in order to eliminate or to reduce the influence of the oxygen concentration on the binding of carbon dioxide; the oxygen concentration necessary for maximum carbon dioxide binding at all carbon dioxide concentrations in the gaseous phase was considered to be attained (cf. exceptions below).

In corresponding experiments with starved yeast, a remarkably smaller amount of bound carbon dioxide was found in the yeast. The examples given in Tables 19 a and b show that the amount of bound carbon dioxide in this yeast clearly increases with increasing oxygen concentration in the gaseous phase.

4. Sources of Error and Correction of Blank Values.

In order to ascertain whether "gaseous equilibrium", *i. e.* a minimum of lag in the gas exchange, prevailed at the moment of tilting, when the method described above was used, experiments were performed at different rates of shaking. Table 20 shows the results of two experiments with baker's yeast under both aerobic and anaerobic conditions, where the apparatuses were shaken at different rates.

Table 20.

Control of "gaseous equilibrium" in the apparatuses at different rates of shaking.

Total amount of CO₂ in $\mu\text{l}.$, obtained after fixing of 1.50 ml. suspension containing 200 mg. baker's yeast with 0.30 ml. p-toluenesulphonie acid. Apparatuses filled with gas during shaking at 96 complete oscillations per minute. Experiments I and II were performed on different yeasts.

Composition of gaseous phase	I		II	
	Rate of shaking 10 min. before tilting (Complete oscillations per minute)			
	64	120	102	130
50 % O ₂ + 25 % CO ₂ + 25 % N ₂	93	95	100	102
25 % CO ₂ + 75 % N ₂ ,	40	40	43	44

There is no significant difference between the pairs of values. The rate of shaking (100 complete oscillations per minute) used in the other experiments can be regarded as sufficient to secure "gaseous equilibrium".

In the determination of the amount of bound carbon dioxide in yeast, a metabolism occurs within the cells, during the period before the tilting of the p-toluenesulphonic acid, which causes pressure changes in the apparatuses. The errors originating from these changes are, however, very small. Table 21 illustrates the order of magnitude of the somewhat varying pressure changes occurring during the 10 minutes immediately before tilting in experiments with a gaseous phase of nitrogen or a mixture of nitrogen and increasing concentrations of oxygen, taking no account of the concentration of carbon dioxide in the mixture. In the presence of higher concentrations of carbon dioxide, somewhat larger pressure changes are obtained in experiments under aerobic conditions than when only mixtures of oxygen and nitrogen are used, a phenomenon which, however, is of minor importance for the estimation of possible errors originating in the pressure changes observed.

Table 21.

Pressure changes in mm. BRODIE solution during the last 10 minutes before tilting of p-toluenesulphonic acid.

200 mg. baker's yeast per apparatus.

Gaseous phase with	Baker's yeast	Starved yeast	Fed yeast	Budding yeast
0 % O ₂	+ 4	+ 1	+ 32	+ 30
15 % O ₂	- 11	- 2	- 25	- 20
25 % O ₂	- 11	- 3	- 40	- 40
50 % O ₂	- 11	- 3	- 30	- 30

The readings and the tilting of p-toluenesulphonic acid were always performed by two persons, more than 6 apparatuses never being used simultaneously. The interval between reading and tilting was 30 seconds at the most. During this period, a pressure change of 1/20 of the values given in the table was obtained. It results from Table 21 that only in experiments with fed and budding yeasts are the errors noticeable. In view of the pressure changes before tilting under anaerobic conditions, the amount of carbon dioxide thus measured in 200 mg. of these yeasts is 1—2 μ l. too

high, while under aerobic conditions it is 1—2 μl . too low. In subsequent determinations, however, the values obtained were not corrected unless otherwise indicated.

Corrections for varying barometric pressure in the different experiments were only performed in the cases of exceptionally low or high barometric pressures. If, for example, the barometric pressure is 15 mm. Hg lower or higher than normal (760 mm. Hg), a deflection of 100 mm. BRODIE solution corresponds to a value which deviates by about 2 μl . from the normal value. This deviation is, however, considerably smaller than the variations of the amount of bound carbon dioxide in the yeast. In contrast to the errors caused by the alterations of pressure before tilting, an error is found in the same series of experiments which tends in the same direction both under aerobic and anaerobic conditions.

A source of error in the determination of the amount of bound carbon dioxide in yeast, primarily noticeable in experiments with a gaseous phase with a low carbon dioxide concentration, is the production of carbon dioxide during endogenous metabolism in the period from the stopping of the gas flow to the moment of tilting. During this period, which lasts for an average of 17 minutes, a concentration of carbon dioxide somewhat lower than 1 % was obtained in the gaseous phase in experiments with baker's yeast under aerobic conditions. The corresponding values for fed and budding yeasts amount to c. 1.5 to 2 %. The change in the carbon dioxide content in the gaseous phase obtained under anaerobic conditions can, however, be neglected. Measurements in the presence of low carbon dioxide concentrations were corrected for the increase in the carbon dioxide concentration in the gaseous phase under aerobic conditions by the construction of graphical mean curves (Figs. 32 a and b).

The blank values determined with a gaseous phase containing carbon dioxide do not permit a quite exact correction of the measurements of carbon dioxide bound in yeast. As emphasized previously, the concentration of the buffer before tilting in the presence of yeast is 0.059 M instead of $M/15$, this latter concentration being employed for the determinations of the blank values. The value of α_{CO_2} in the yeast cell presumably deviates from the α values of the buffer; this fact causes a discrepancy between the total amount of carbon dioxide dissolved in the suspension before tilting and the amount dissolved in the $M/15$ buffer used in the determination of the blank values, as described above. It

may, furthermore, be assumed that the solubility of carbon dioxide in the mixture of buffer and p-toluenesulphonie acid is influenced by the content of fixed yeast. The supposition that α_{CO_2} after tilting does not deviate markedly from the α value previously determined for the acid-buffer mixture is supported by the fact that a difference in the amount of carbon dioxide measured in the yeast cannot be detected when different vessel volumes are used (15 and 17 ml.).

In order to determine the true blank values to be subtracted from the amount of carbon dioxide obtained in the presence of yeast in a carbon dioxide containing atmosphere, experiments were performed with varying amounts of yeast. If the presence of yeast does not influence the blank values measured previously, the amount of carbon dioxide measured in the yeast should be a linear function of the amount of yeast. Since, with varying amounts of yeast, the possible effect of the yeast content upon α in the final mixture cannot be assumed to be constant, a minor deviation from the straight line can be expected. If the values obtained are included in a coordinate system, the amounts of carbon dioxide expelled (in $\mu l.$) being plotted as ordinates against the quantities of yeast (in mg.) as abscissae, the tangent to the curve at the point corresponding to 200 mg. of yeast should meet the y-axis at the corrected blank value.

The measurements were performed on baker's yeast and starved yeast both under aerobic and anaerobic conditions in the presence of 50 % carbon dioxide in the gaseous phase. In all cases, the concentration of the buffer was 0.059 *M* (corrected for different amounts of intercellular water originating from different quantities of yeast employed). Fig. 24 shows the curves obtained from which the probable true blank value was estimated graphically. Despite the difference in the amounts of bound carbon dioxide in the yeasts employed, the same blank value should be obtained in all cases. It is seen from the curves that this was the case. If the measurements were performed under anaerobic conditions, the amounts of bound carbon dioxide in the cells were small as compared to the blank value. This contributes to a more accurate determination of the latter. This result is based not only on the weak slope of the curve towards the y-axis, but also on the fact that the small amount of carbon dioxide within the cells does not noticeably increase the carbon dioxide pressure in the gaseous phase after tilting. The blank value obtained from the

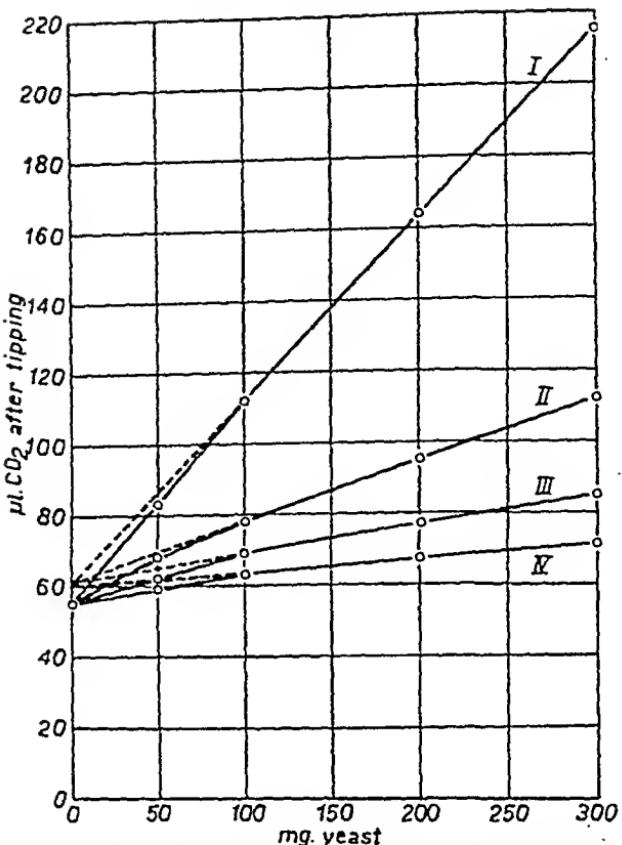


Fig. 24. Graphical determination of the blank value in measurements of bound CO₂ in yeast. 0.30 ml. 3.64 M p-toluenesulphonic acid + 1.50 ml. suspension with varying amounts of yeast in 0.059 M Na-SS buffer, pH 5.1. The tangents of the curves for total amount of CO₂, through the points corresponding to 200 mg. yeast meet on the y-axis at the blank value valid for 200 mg. yeast and a gaseous phase with 50 % CO₂ (= 61 μl.).

- (I) In the presence of baker's yeast. Gaseous phase with 50 % O₂ + 50 % CO₂.
- (II) In the presence of starved yeast. Gaseous phase with 50 % O₂ + 50 % CO₂.
- (III) In the presence of baker's yeast. Gaseous phase with 50 % N₂ + 50 % CO₂.
- (IV) In the presence of starved yeast. Gaseous phase with 50 % N₂ + 50 % CO₂.

measurements was 61 μl. carbon dioxide, valid in the presence of 200 mg. of yeast and 50 % carbon dioxide in the gaseous phase. The blank values valid at different carbon dioxide concentrations were obtained from the above value, assuming proportionality with the carbon dioxide concentration (cf. Table 16).

Using Na-SS buffers with different pH values or K-SS and Ca-SS buffer, pH 5, the blank value was determined in an analogous manner. In buffers with pH about 5.5 and 6, at equilibrium with a gaseous phase containing carbon dioxide, the pH decreases to varying extents with different concentrations of carbon dioxide (cf. Table 3). Determinations of the blank value in experiments

with these buffers were therefore performed, using the same concentration of carbon dioxide as in the determinations of bound carbon dioxide in yeast. In experiments with buffers of different pH values, the calculations of the bound carbon dioxide were made with the same vessel constants as in experiments with Na-SS buffer, pH 5.1. Errors involved in this procedure should be negligible.

C. Determinations.

1. Treatment of the Measurements.

In the following description, the amounts of bound carbon dioxide in different yeasts under various physiological conditions are generally represented by curves. The values given are the means of three simultaneous determinations and are corrected by the blank value for the concentration of carbon dioxide employed.

The carbon dioxide binding was examined partly at a constant oxygen concentration and different concentrations of carbon dioxide in the gaseous phase, and partly at constant carbon dioxide concentration and different concentrations of oxygen. For practical reasons, the number of determinations carried out simultaneously was not sufficient to establish accurately the complete courses of the curves. The amounts of carbon dioxide or oxygen in the gas mixtures were therefore varied in such a way as to give measurements either scattered over the whole range of the curve or collected within a certain region, where the variations in the gas mixtures had to be made within narrow limits for a precise determination of the curve. In drawing the curves, the courses of adjacent regions in other experiments were also taken into consideration. The different measurements refer to yeasts available at various seasons of the year. The scattering of the values observed in different experiments may therefore largely be explained by the previously discussed variations in the initial yeast.

2. Acid-labile Bound Carbon Dioxide in Baker's Yeast. Effect of Aerobic Starvation, Alteration of the pH value in the Suspension Medium, and Irradiation.

Fig. 25 represents the amount of carbon dioxide bound in baker's yeast and in starved yeast in the presence of 50 % oxygen and different concentrations of carbon dioxide in the gaseous

phase. Table 18 a has already shown that, in experiments on baker's yeast, the amount of bound carbon dioxide strongly increases with increasing concentration of carbon dioxide (curves I in Fig. 25). When, before the start of the experiment, baker's yeast is shaken under aerobic conditions for 30 minutes, a minor quantity of carbon dioxide is bound by the yeast (curve II).

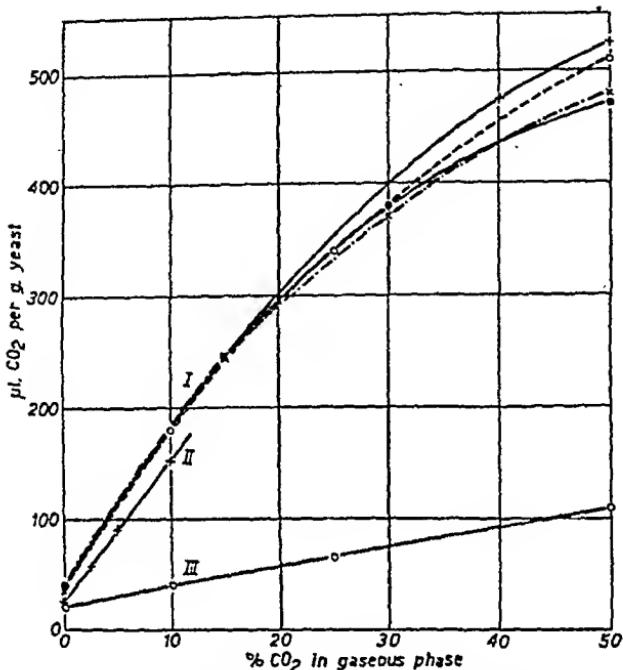


Fig. 25. Bound CO₂ in baker's yeast and starved yeast. Gaseous phase with 50 % O₂, and varying amounts of CO₂.

- (I) Baker's yeast.
- (II) Baker's yeast shaken in air for 30 min.
- (III) Starved yeast. (The values are lower than are usually obtained, e.g. the mean value for a gaseous phase with 50 % O₂ + 50 % CO₂ is 145 μl. per g. yeast.)

With a gaseous phase containing 0—10 % carbon dioxide, this curve is practically linear. As already emphasized previously, the amount of bound carbon dioxide in the cells after starvation of the yeast is markedly less than in baker's yeast, increasing but slowly with increasing concentration of carbon dioxide in the gaseous phase (curve III).

The amount of carbon dioxide bound in baker's yeast under both aerobic and anaerobic conditions decreases during shaking of the suspensions in *M/15 Na-SS* buffer, pH 5.1. Table 22 gives the amounts of carbon dioxide determined in the presence of 50 % carbon dioxide in the gaseous phase.

Table 22.

Bound CO₂ in μl. per g. baker's yeast shaken in air at 25° C. for different periods before the beginning of the determinations.

Composition of gaseous phase	Time of aeration (in hours)					
	0	3/4	2 1/2	6	22	46
50 % CO ₂ + 50 % O ₂	495	390	325	—	120	—
50 % CO ₂ + 50 % N ₂	80	—	—	55	35	25

In order to investigate whether the carbon dioxide binding in the presence of high concentrations of carbon dioxide in the gaseous phase and under aerobic conditions was reversible, two suspensions of baker's yeast were shaken in small culture flasks for 45 minutes at 25° C. before the commencement of the measurements. One of these suspensions was shaken with a gaseous mixture of 50 % O₂ + 50 % N₂, the other with 50 % O₂ + 50 % CO₂. The yeasts were centrifuged off immediately, washed twice with distilled water, and suspended in Na-SS buffer, pH 5.1. The amounts of carbon dioxide bound in the yeasts were determined in a gaseous phase containing 0 % and 50 % carbon dioxide. The results of these experiments are given in Table 23.

Table 23.

Reversibility of CO₂-binding in baker's yeast.

Composition of gaseous phase	μl. CO ₂ per g. yeast	
	Yeast pretreated with 50 % O ₂ + 50 % N ₂	Yeast pretreated with 50 % O ₂ + 50 % CO ₂
50 % O ₂ + 50 % N ₂	30	35
50 % O ₂ + 50 % CO ₂	395	430

The table makes it clear that carbon dioxide bound by the yeast during pretreatment at high carbon dioxide pressure is liberated anew during subsequent shaking in a CO₂-free gaseous phase. The residual amount is practically the same in yeast pretreated in the absence of carbon dioxide. The amount of carbon dioxide bound in experiments with a gaseous phase containing 50 % carbon dioxide is somewhat greater in yeast pretreated in the presence of carbon dioxide than in the control experiment. This difference may be due to an increased metabolism during

pretreatment in an atmosphere containing carbon dioxide, and to a presumably increased pH of the protoplasm (cf. Chapter V).

The effect of the pH of the suspension medium on the amount of bound carbon dioxide in the yeast was examined, using Na-SS buffers of different pH values (cf. Chapter II, A). The measurements were performed on baker's yeast with gaseous phases containing 50 % O₂ + 25 % CO₂ + 25 % N₂ and 50 % O₂ + 50 % CO₂. The values were calculated on the basis of blank values determined graphically for different buffers in the presence of yeast and at the carbon dioxide pressures of the actual experiments. Fig. 26 shows the dependence of carbon dioxide binding on the pH of the suspension medium, when the composition of the gaseous phase was 50 % O₂ + 25 % CO₂ + 25 % N₂. The pH values plotted in the curves refer to the initial values of the buffer and thus are not corrected for the rather small decrease in pH obtained in the suspensions at equilibrium with a gas mixture containing 25 % carbon dioxide. The values for bound carbon dioxide found at pH 5.1 are somewhat lower than those generally obtained. The points indicated with similar signs represent measurements performed simultaneously on the same yeast.

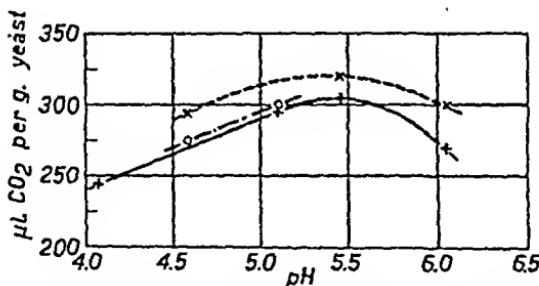


Fig. 26. Bound CO₂ in baker's yeast suspended in Na-SS buffer with different pH values. Gaseous phase with 50 % O₂ + 25 % CO₂ + 25 % N₂.

It results from the experiments that the amount of bound carbon dioxide is dependent on the pH of the suspension medium. Maximum binding is obtained at about pH 5.0—5.5. Similar results were obtained in experiments on baker's yeast with a gaseous phase containing 50 % O₂ + 50 % CO₂.

The dependence of the carbon dioxide binding on the pH of the suspension medium may also be estimated without graphical determination of the different blank values. When we base the corrections on the total amounts of carbon dioxide expelled under

anaerobic conditions from suspensions of starved yeast in corresponding buffers, we find a dependence on pH identical with that described above. Since the ability of starved yeast to bind carbon dioxide under anaerobic conditions is very small, a possible change in the amount of carbon dioxide bound in the cells during variation of the pH in the suspension medium can affect but slightly the total amount of carbon dioxide expelled. At different pH values this amount is increased in almost equal amounts and all values of bound carbon dioxide thus corrected are consequently somewhat too low.

Investigations were carried out with the aim of elucidating whether the amount of carbon dioxide bound in baker's yeast varied when the treatment was performed in the dark and during exposure to visible light. Six apparatuses were irradiated with three 200 W lamps which were cooled with running water in special troughs with glass windows and placed in the thermostat. The irradiation was maintained from the beginning of the passage of gas until the end of the experiment. Six apparatuses shaken simultaneously in the dark were wrapped in black cloth. Table 24 shows the values from two such experiments at 25° C. with 200 mg. of yeast in Na-SS buffer, pH 5.1, per apparatus. There is obviously no significant difference between the carbon dioxide binding in visible light and that in the dark.

Table 24.

Bound CO₂ in baker's yeast in light and in dark.

Composition of gaseous phase	$\mu\text{l. CO}_2 \text{ per g. yeast}$	
	In light	In dark
25 % CO ₂ + 75 % N ₂	60	60
25 % CO ₂ + 10 % O ₂ + 65 % N ₂	200	210
25 % CO ₂ + 50 % O ₂ + 25 % N ₂	325	330

The change in the amount of carbon dioxide bound by baker's yeast and starved yeast in experiments performed with a gaseous phase containing different amounts of oxygen, but constant concentrations of carbon dioxide, is shown in Fig. 27. Measurements were carried out on baker's yeast with gaseous phases containing 10, 25 and 50 % carbon dioxide. In all cases a strong increase in the amount of bound carbon dioxide was observed when the

oxygen concentration was raised from 0 % to c. 20 %. A further oxygen increase in the gaseous phase, however, affected only slightly the amount of carbon dioxide bound. In experiments on starved yeast (curve I), it was also found that the amount of bound carbon dioxide was dependent on the concentration of oxygen in the gaseous phase.

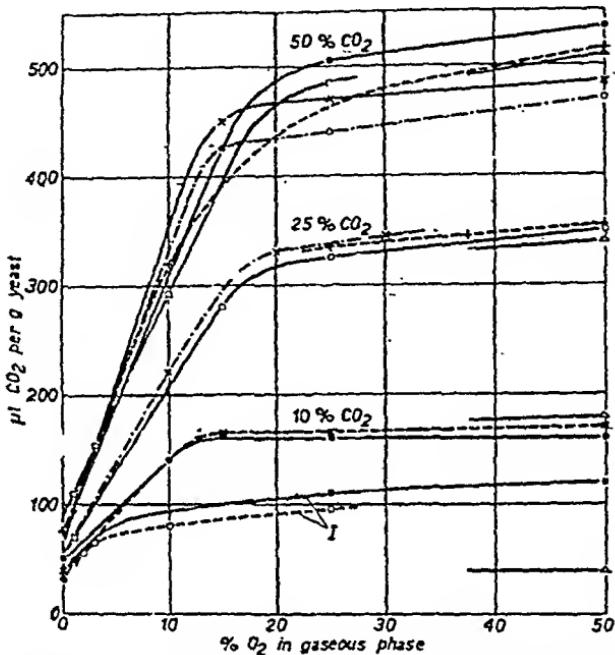


Fig. 27. Bound CO₂ in baker's yeast. Gaseous phase with constant CO₂ concentration (10, 25, and 50 % CO₂) and varying amounts of O₂. The points Δ refer to measurements on the same yeast sample with 0, 10, 25, and 50 % CO₂ in the gaseous phase.

(I) Bound CO₂ in starved yeast. Gaseous phase with 50 % CO₂.

3. Carbon Dioxide in Fed Yeast. Reversibility of Starvation Effect.

Fig. 28 shows the amount of carbon dioxide bound in fed yeast in experiments with gaseous phases containing 50 % oxygen and different amounts of carbon dioxide. The curves indicate that, for gaseous phases containing 0—30 % carbon dioxide, the amounts of bound carbon dioxide are approximately equal to those obtained in experiments on baker's yeast. At higher concentrations of carbon dioxide, however, much more carbon dioxide is bound in fed yeast than in baker's yeast under corresponding conditions. An increase in the carbon dioxide concentration from

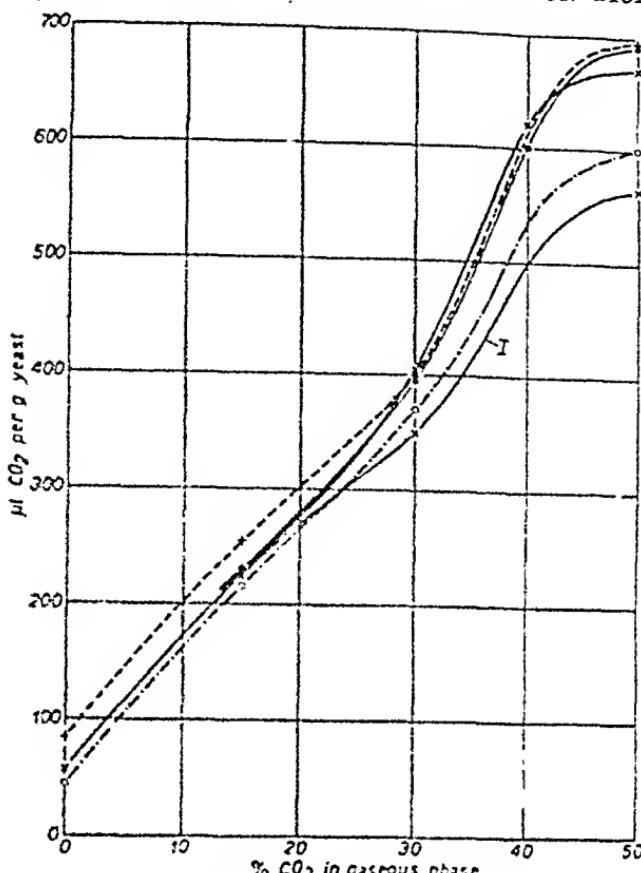


Fig. 28. Bound CO₂ in fed yeast. Gaseous phase with 50 % O₂ and varying amounts of CO₂.

(I) Yeast fed after starvation. Other curves: fed yeast.

30—40 % involves, moreover, a definitely more rapid increase in the amount of bound carbon dioxide than does a corresponding change at a lower concentration of carbon dioxide in the gaseous phase. This might be attributed to the fact that it is only at high carbon dioxide concentrations that fed yeast binds the maximum amount of carbon dioxide in the presence of 50 % oxygen. The measurements represented in Fig. 29 confirm this supposition, as they clearly show that for gaseous phases containing 10 % and 25 % carbon dioxide the amount of bound carbon dioxide in fed yeast is greatest in the presence of about 30 % oxygen. In the presence of 50 % carbon dioxide, however, maximum binding is first reached at an oxygen concentration of 40—50 %.

The curves for bound carbon dioxide in fed yeast are thus markedly different from those obtained in experiments on baker's yeast with respect to variations in the concentration of both carbon dioxide and oxygen in the gaseous phase. This effect of

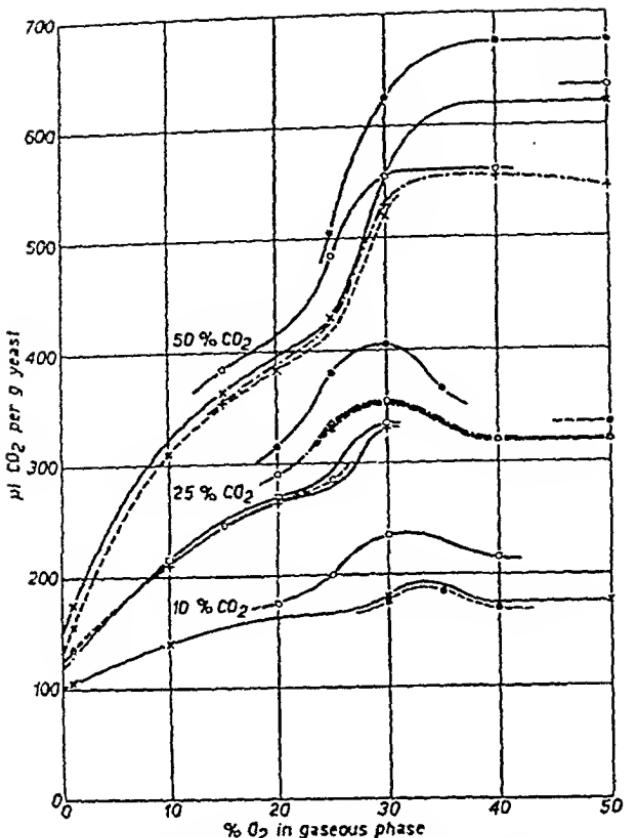


Fig. 29. Bound CO₂ in fed yeast. Gaseous phase with constant CO₂ concentration (10, 25, and 50 % CO₂) and varying amounts of O₂.

feeding on carbon dioxide binding also appears from measurements on a yeast, fed after starvation, with a gaseous phase containing 50 % oxygen and different amounts of carbon dioxide (Fig. 28, curve I). The reduced power of binding carbon dioxide, typical of starved yeast, is obviously reversible!

Experiments were also carried out on baker's yeast fed under practically anaerobic conditions. (Initial composition of gaseous phase 99 % N₂ + 1 % O₂.) With a gaseous phase containing 25 % carbon dioxide and 0—20 % and 50 % oxygen, the same values for bound carbon dioxide were found as in experiments on yeast pretreated with glucose under aerobic conditions. However, the maximum seen in Fig. 29 in the presence of 20—40 % oxygen is absent, the course of the curve being fairly even. It is therefore assumed that the maximum in bound carbon dioxide, observed during a variation in the oxygen concentration at a constant, moderate carbon dioxide concentration, originates from pretreatment under aerobic conditions.

4. Carbon Dioxide in Budding Yeast.

Fig. 30 shows the amounts of carbon dioxide bound in budding yeast with gaseous phases containing 50 % oxygen and different amounts of carbon dioxide. When these values are compared with those for carbon dioxide bound under corresponding con-

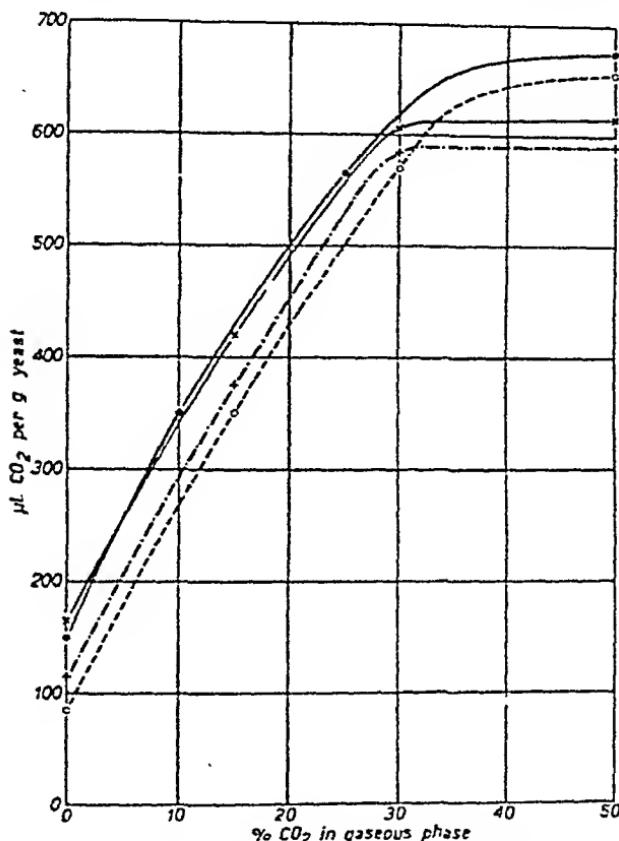


Fig. 30. Bound CO₂ in budding yeast. Gaseous phase with 50 % O₂ and varying amounts of CO₂.

ditions in baker's yeast and fed yeast, it is clear that budding yeast binds much larger amounts of carbon dioxide than the other yeasts. When the gaseous phase contains 50 % carbon dioxide and 40—50 % oxygen, only fed yeast is capable of binding as much carbon dioxide as budding yeast under analogous conditions.

The dependence of carbon dioxide binding on the oxygen concentration (Fig. 31) indicates, however, that the amount of carbon dioxide bound in budding yeast is greater than in baker's yeast and in fed yeast only if about 50 % oxygen is present in

the gaseous phase. As in experiments on fed yeast, after a change in the oxygen concentration, a maximum is observed in the capacity for binding carbon dioxide which, however, is not reached unless about 50 % oxygen is present in the gaseous phase in experiments with 10, 25 and 50 % carbon dioxide. Experiments

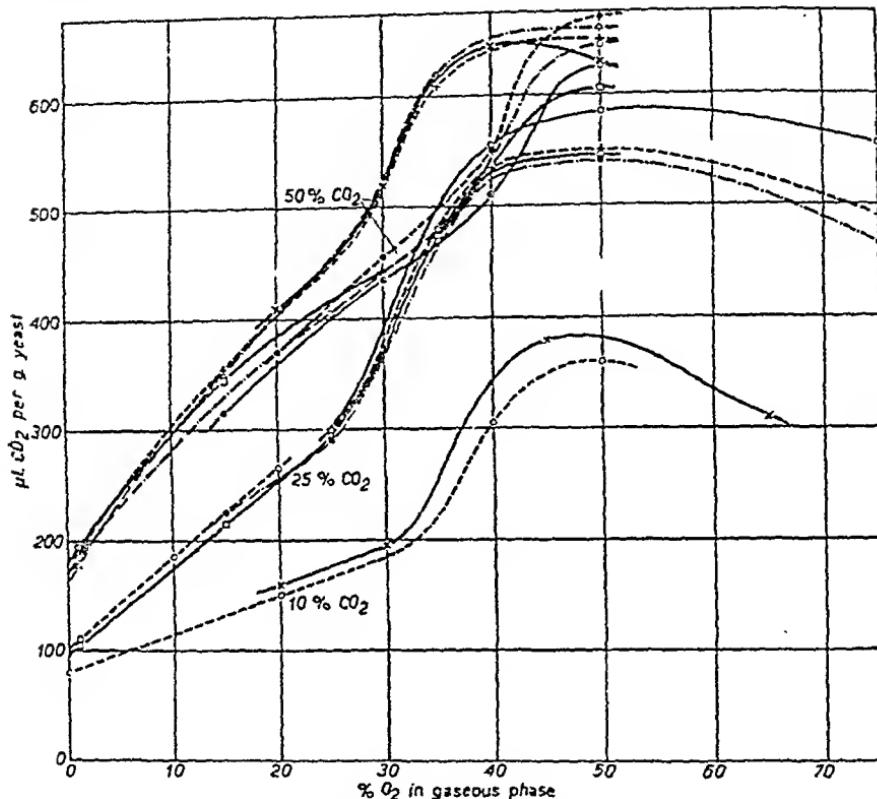


Fig. 31. Bound CO₂ in budding yeast. Gaseous phase with constant CO₂ concentration (10, 25, and 50 % CO₂) and varying amounts of O₂.

with gaseous phases containing 50 % carbon dioxide showed a marked variation in the values of bound carbon dioxide for different yeast samples in the presence of 30–40 % oxygen. In a gas mixture containing 50 % carbon dioxide and 50 % nitrogen, the quantity of bound carbon dioxide in budding yeast is clearly greater than the corresponding values found for baker's yeast and fed yeast. The curves illustrating the capacity of budding yeast for binding carbon dioxide at different concentrations of oxygen and carbon dioxide in the gaseous phase thus deviate characteristically from all other curves previously obtained for both baker's yeast and fed yeast.

5. Corrected Graphical Mean Curves.

For more ready comparison of the values obtained for the different yeasts, Figs. 32 a and b show curves drawn with graphically determined mean values of the above discussed measurements with gaseous phases containing 50 % oxygen and different amounts of carbon dioxide (a) and 25 % carbon dioxide and different amounts of oxygen (b). The curves have been corrected for possible errors due to pressure changes occurring during the period between the reading of the manometer before tilting the acid and the moment of tilting, and for increases in the carbon dioxide pressure in the apparatuses consequent upon the carbon dioxide production in the interval between the passage of gas and the tilting. No correction has been made for the increase in the initial amount of protein obtained during the preparation of budding yeast, since this was regarded as unnecessary (cf. Chapter II, C). A reduction of all values for budding yeast by, for example, 10 % does not significantly affect the slopes of the curves.

6. Effect of Drying on the Yeast.

The amount of bound carbon dioxide was also determined in suspensions of dried yeast treated in different manners. The dried yeast employed had been stored in the refrigerator for seven months. The determinations were performed only with a gaseous phase containing 50 % oxygen and 50 % carbon dioxide. For purposes of comparison with the amounts of carbon dioxide bound under corresponding conditions in the other yeasts, the values are given in μl . per gram of calculated wet weight.

After dried yeast is washed, the metabolism is changed owing to the loss of certain plasma constituents (see Chapter II, C). In the determination of the amount of carbon dioxide bound in dried yeast, different results were also obtained, according to whether the yeast was washed before the experiment or not. In the unwashed yeast, 75 μl . bound carbon dioxide was found per gram of yeast, while in yeast washed three times 90 μl . bound carbon dioxide was obtained per gram of yeast. Both values indicate that the amount of carbon dioxide bound in dried yeast, in the presence of 50 % oxygen + 50 % carbon dioxide, is somewhat smaller than that bound in starved yeast under corresponding conditions (mean: 145 μl . CO_2 per g. starved yeast). The

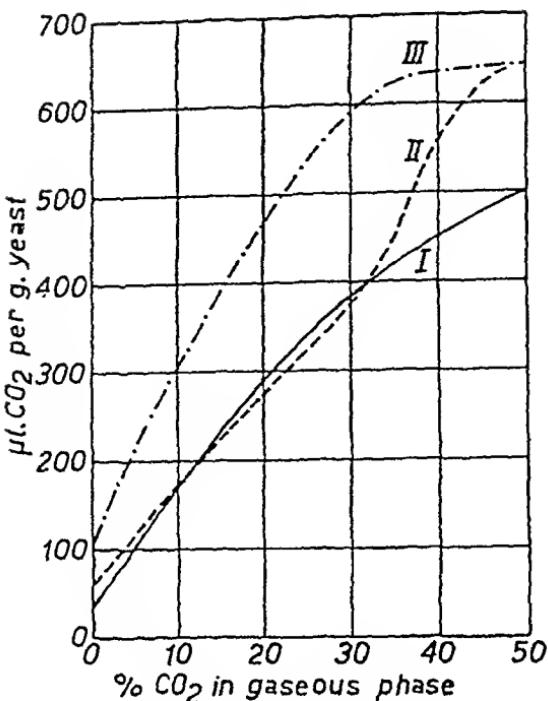


Fig. 32 a. Corrected graphical mean curves for bound CO_2 . Gaseous phase with 50 % O_2 and varying amounts of CO_2 .
 (I) Baker's yeast. (II) Fed yeast. (III) Budding yeast.

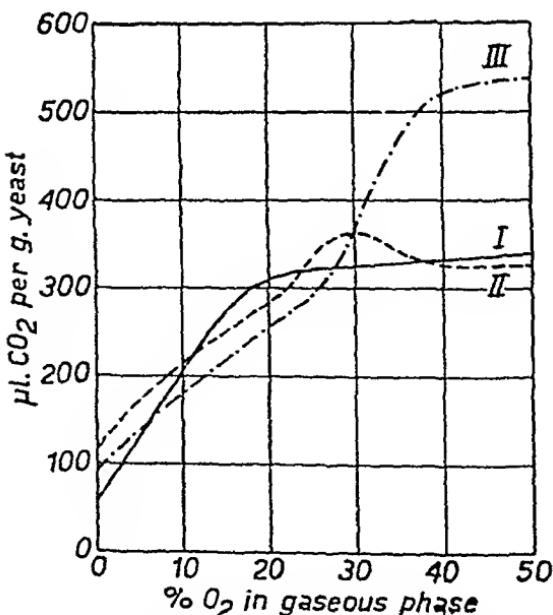


Fig. 32 b. Corrected graphical mean curves for bound CO_2 . Gaseous phase with 25 % CO_2 and varying amounts of O_2 .
 (I) Baker's yeast. (II) Fed yeast. (III) Budding yeast.

dried yeast was shaken in $M/10$ Na-SS buffer at 25° C. in air for 20 hours and was subsequently washed. After this treatment, the amount of bound carbon dioxide was increased to $120 \mu\text{l}$. per gram of yeast. Addition of glucose (750 mg. per 280 mg. dried yeast) during shaking, half at the start of the aerobic treatment and half one hour before the end of the pretreatment, gave a corresponding value for the subsequently washed yeast of $165 \mu\text{l}$. carbon dioxide per gram of yeast. The restituting effect of shaking under aerobic conditions on dried yeast discussed previously, which appears with special distinctness after simultaneous addition of glucose, is thus found also in the study of the capacity of originally dried yeast for binding carbon dioxide. The amount of bound carbon dioxide in pretreated dried yeast, however, never reaches values as high as those found in baker's yeast or yeast fed after previous starvation (cf. Figs. 25 and 28).

7. Influence of Cell Poisons on the Capacity of the Yeast for Binding Carbon Dioxide.

From the above observations concerning the amounts of carbon dioxide bound in different yeasts at varying concentrations of oxygen and carbon dioxide in the gaseous phase, it results that the binding of carbon dioxide invariably increases strongly with increase in the oxygen concentration to a certain limit and that a change in the endogenous metabolism of the yeast influences characteristically the dependence of CO_2 -binding on the composition of the gaseous phase. It should be emphasized that the magnitude of the endogenous metabolism generally cannot be regarded as related to the capacity for binding carbon dioxide under various conditions. During shaking of, for example, baker's yeast in buffer under aerobic conditions, its power of binding carbon dioxide is rapidly reduced, while the endogenous monomolecular respiration simultaneously decreases. However, in suspensions of dried yeast in which, together with restituting processes, a metabolism takes place under aerobic conditions which is relatively high as compared with that of starved yeast, the capacity for binding carbon dioxide is certainly very low, although the respiratory enzyme system in dried yeast is greatly impeded. A comparison between the amounts of carbon dioxide bound by baker's yeast, fed yeast and budding yeast in a gaseous phase containing, for example, 20 % oxygen + 25 % carbon

dioxide + 65 % nitrogen, shows that baker's yeast, despite its lower endogenous metabolism, binds somewhat more carbon dioxide than does fed or budding yeast (Fig. 32 b).

It is tempting to conclude that the different powers of yeast to bind carbon dioxide at different concentrations of oxygen in the gaseous phase must mainly be attributed to properties of the protoplasm which are regulated by the respiratory system. The state of this system and its power for acting together with other enzymic systems in the protoplasm may thus be of importance for the carbon dioxide binding. Inhibition or activation of the enzymic systems, directly or indirectly affecting carbon dioxide binding, need not be accompanied by corresponding variations in the intensity of the endogenous respiration, which is partly dependent on the supply or mobilization of endogenous substrate. In attempts to check the validity of this hypothesis, an examination was made of the power of binding carbon dioxide shown by baker's yeast after this had been treated with certain cell poisons which are supposed to affect the respiratory system.

The experiments were performed on baker's yeast washed twice with distilled water. 60 ml. suspension containing 1 g. yeast in 0.081 *M* Na-SS buffer, pH 5.1, and different concentrations of sodium azide, sodium fluoride or potassium cyanide were shaken in 450 ml. culture flasks at 25° C. for 30 minutes. The suspensions were then cooled, and the yeast was centrifuged off. In order to preserve as far as possible the effect of pretreatment, the yeast was not washed after centrifuging, but was suspended immediately in Na-SS buffer, pH 5.1, for the determination of bound carbon dioxide. During the period required for pipetting the suspensions into the vessel, filling the apparatuses with gas, and reading before the tilting of p-toluenesulphonic acid, small amounts of the cell poisons remained in the suspensions. These quantities were, however, assumed not to affect the blank values. The determinations were performed with a gaseous phase containing 50 % oxygen + 25 % carbon dioxide + 25 % nitrogen. Table 25 summarizes the values obtained.

After the suspensions had been shaken, without addition of cell poisons, for 30 minutes in air before the amounts of bound carbon dioxide were determined, there was found, in analogy to previous observations, a smaller amount of bound carbon dioxide, *viz.* 230—250 μ l. per gram of yeast, than in the untreated baker's yeast which, under corresponding experimental condi-

Table 25.

Bound CO₂ in baker's yeast pretreated in the presence of different concentrations of NaN₃, NaF and KCN.
Gaseous phase containing 50 % O₂ + 25 % CO₂ + 25 % N₂.

NaN ₃		NaF		KCN	
Concentration during pretreatment (millimol/l.)	μl. CO ₂ per g. yeast	Concentration during pretreatment (millimol/l.)	μl. CO ₂ per g. yeast	Concentration during pretreatment (millimol/l.)	μl. CO ₂ per g. yeast
0	245	0	230	0	250
0.09	290	31	270	4	325
0.29	95	51	345	21	125
1.00	40	72	20	50	90

tions, binds c. 340 μl. carbon dioxide per gram of yeast. As is seen from Table 25, pretreatment of the yeast with moderate concentrations of azide, fluoride or cyanide gave rise to an increase in the ability to bind carbon dioxide. Higher concentrations of the poisons, however, greatly inhibited the binding capacity.

BOREI (1942) has shown that under the action of azide and fluoride a similar change occurs in the endogenous respiration of baker's yeast. This author found an activation and an inhibition of the endogenous respiration at somewhat lower concentrations of the cell poisons than were required in the above experiments on the CO₂-binding capacity. Higher concentrations of azide and fluoride are needed for the pretreatment of the yeast in the latter experiments, as the partly reversible effect on the yeast must be preserved during the subsequent treatment of the centrifuged cells. For the same reason, in the pretreatment of the cells with cyanide, higher cyanide concentrations were required than were necessary for an inhibition of the endogenous respiration. That the effect of azide on the ability to bind carbon dioxide was reversible, could be shown by experiments on yeast pretreated with sodium azide in the concentration 1.0 millimoles per litre. As stated above, the centrifuged yeast exhibited a markedly reduced power of binding carbon dioxide (gaseous phase containing 50 % O₂ + 50 % CO₂). After the yeast was washed twice with water, a considerably greater amount of bound carbon dioxide was found than in yeast untreated with azide.

According to KEILIN and HARTREE (1939), azide combines with cytochrome a₃, a process causing a complete inhibition of

the oxidation of the cytochromes in heart muscle extract. BOREI (1942) has pointed out, moreover, that with azide and fluoride, a maximum inhibition of both the monomolecular and the constant endogenous respirations can be obtained, which are both supposed chiefly to occur over the cytochrome oxidase—cytochrome system. The inhibiting effect of cyanide on the respiratory mechanism may be attributed to the reaction of cytochrome oxidase with hydrogen cyanide (cf. WARBURG 1934, and TAMAYA and KUBO 1938). LINDAHL (1940) showed that potassium cyanide in concentrations of 2 millimoles per litre strongly inhibited the endogenous respiration of baker's yeast while a high respiratory quotient was simultaneously obtained. The latter, as well as the oxygen consumption of cyanide-poisoned cells, was higher in pure oxygen than in air. PETT's (1936) observation that cyanide added to top yeast partly disappeared was also confirmed by LINDAHL, who found that cyanide was still not oxidized completely at high oxygen pressure.

The present observations regarding the influence of azide, fluoride and cyanide on the amount of carbon dioxide bound in baker's yeast strongly support the view that the increased ability of yeast to bind carbon dioxide under aerobic conditions is largely dependent on the function of the respiratory enzymes, here presumably that of the cytochrome oxidase—cytochrome system.

The amount of bound carbon dioxide was also determined in baker's yeast pretreated with glucose in the presence of cysteine. The effect of cysteine on the aerobic turnover of glucose by yeast was examined by QUASTEL and WHEATLEY (1932) who were able to show that, in the presence of cysteine, aerobic fermentation occurred simultaneously with the appearance of a respiratory inhibition. This observation was recently confirmed by RUNNSTRÖM and SPERBER (1938 b) in their investigations on Swedish baker's yeast. They found, in the presence of cysteine in a concentration of 2.1 millimoles per litre, in addition to strong aerobic fermentation and impeded respiration, a largely reduced storage of carbohydrates in the yeast (see also RUNNSTRÖM, BOREI and SPERBER 1939, and RUNNSTRÖM and BRANDT 1941).

In the following experiments, yeast was treated, in the presence of glucose and cysteine, in the same manner as in the preparation of fed or budding yeast. The initial concentration of cysteine in the suspension was 0.84 millimoles per litre (cysteine hydrochloride neutralized with hydroxide to pH 5). The yeast

obtained had a distinct odour of hydrogen sulphide. After the pretreatment, only few buds were observed. The volutine grains were clearly spread over the protoplasm in the same way as in fed yeast. Iodine staining of the cells gave a positive glycogen reaction which, however, was noticeably weaker than that in fed yeast. Figs. 33 and 34 show the amounts of carbon dioxide bound in cysteine-treated yeast.

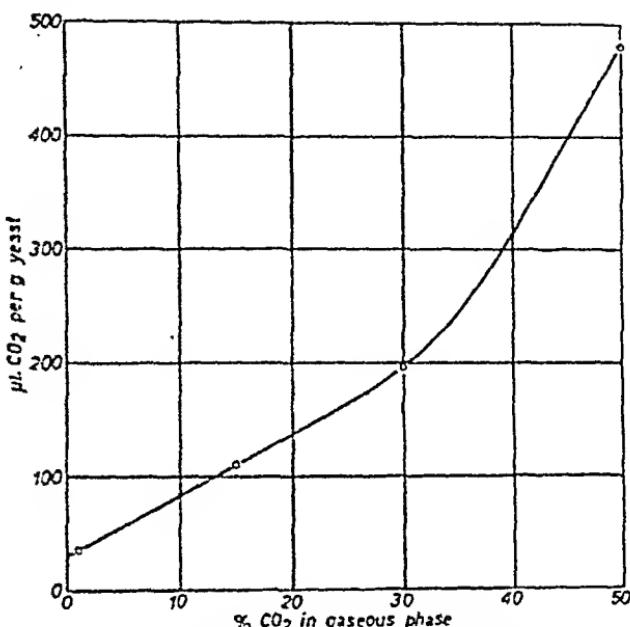


Fig. 33. Bound CO₂ in baker's yeast pretreated with glucose in the presence of 0.84 millimol/l. cysteine. Gaseous phase with 50 % O₂ and varying amounts of CO₂.

In experiments with a gaseous phase containing 50 % oxygen and different amounts of carbon dioxide (Fig. 33), in the presence of up to 30 % carbon dioxide, it was observed that in cysteine-treated yeast the amount of bound carbon dioxide was rather small, increasing rapidly with further increase in the carbon dioxide concentration. The increased power of yeast for binding carbon dioxide in the presence of 50 % oxygen and 50 % carbon dioxide in the gaseous phase is very probably associated with an activation of the endogenous respiration induced by this concentration of carbon dioxide. This is suggested by the fact that, at 50 % carbon dioxide, a considerable pressure reduction is observed in the apparatuses before the tilting of the p-toluenesulphonic acid.

Estimations in the presence of 25 % carbon dioxide and different amounts of oxygen in the gaseous phase (Fig. 34, curve I)

indicate that, in the presence of only 10 % oxygen, the amount of bound carbon dioxide is inconsiderably lower than for higher concentrations of oxygen in the gaseous phase.

Experiments were also carried out on yeast fed in the presence of 4.2 millimoles per litre cysteine (five times the amount employed in the preceding experiment). The yeast obtained smelled faintly of hydrogen sulphide even after being washed three times with cold water. Scarcely any buds were developed. The glycogen reaction was weakly positive. Endogenous respiration was

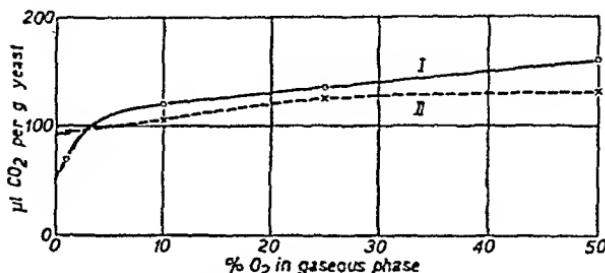


Fig. 34. Bound CO₂ in baker's yeast pretreated with glucose and cysteine. Gaseous phase with 25 % CO₂ and varying amounts of O₂.

(I) Yeast fed in the presence of 0.84 millimol/l. cysteine.
(II) " " " " " 4.20 millimol/l. "

greatly inhibited. The amount of carbon dioxide bound in this yeast at a carbon dioxide concentration of 25 % in the gaseous phase appears from curve II in Fig. 34. This curve shows that an increase in the concentration of oxygen in the gaseous phase, even at low concentrations of this gas, causes only a slight increase in the amount of carbon dioxide bound in the yeast.

As in the experiments where the respiration was impeded by various typical cell poisons, strikingly small amounts of bound carbon dioxide were found after feeding the yeast in the presence of cysteine under aerobic conditions. This was presumably due to the inhibitory effect on the respiration of cysteine or of the hydrogen sulphide evolved.

8. Influence of Potassium and Calcium Ions.

In the investigations so far described, only Na-SS buffers have been used as suspension media. Since the yeast membrane is practically impermeable to sodium ions, the concentration of alkali ions in the protoplasm in the above experiments may be supposed not immediately to be affected by the sodium ions

present in the suspension medium. The potassium and calcium ions of the protoplasm can, however, leave the yeast cells and exchange with ions in the suspension medium (GENAUD 1929, CONWAY, O'BRIEN and BOYLE 1941. Cf. also the review given by WILBRANDT 1938). PULVER and VERZÁR (1940) pointed out, moreover, that the distribution of potassium between the protoplasm of yeast and the suspension medium was changed during the turnover of sugar. As the content of alkali ions in the protoplasm presumably influences the pH of the cell content, it was of interest to investigate whether the ability to bind carbon dioxide was influenced by potassium and calcium ions in the suspension medium. Experiments were performed, using both the K-SS buffer ($M/15$, pH 5.17) and the Ca-SS buffer ($M/15$, pH 4.93 and 5.08) described in Chapter II. Parallel determinations were performed with the aid of a Na-SS buffer, pH 5.14.

The blank values in experiments with 200 mg. of yeast in K-SS or Ca-SS buffer were estimated in the same manner as previously described for the Na-SS buffer. In employing a K-SS

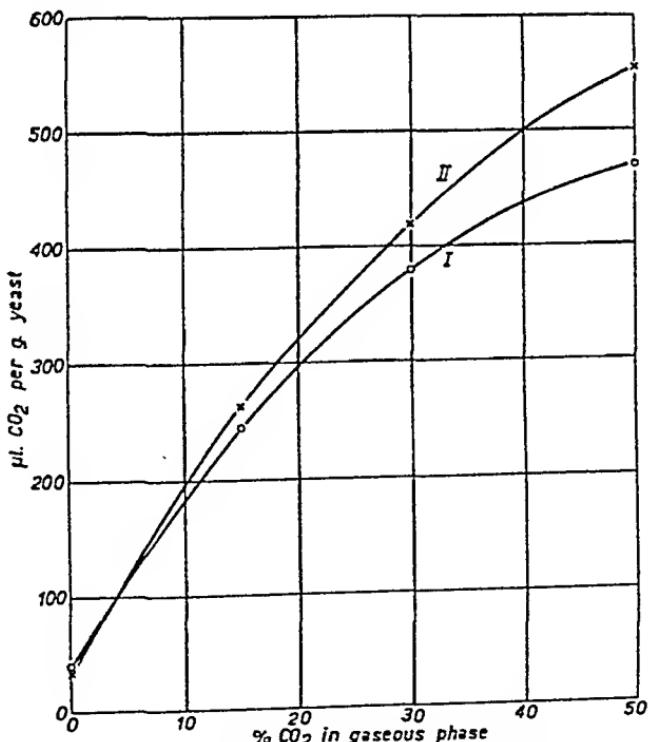


Fig. 35. Bound CO₂ in baker's yeast. Gaseous phase with 50 % O₂ and varying amounts of CO₂.

- (I) Suspension in 0.059 M Na-SS buffer, pH 5.14.
- (II) " " 0.059 M K-SS buffer, pH 5.17.

buffer, the blank value in the presence of 50 % carbon dioxide in the gaseous phase was 63 μl , or 2 μl . higher than the value for the Na-SS buffer (61 μl .). Similar differences were also obtained in numerous estimations of the uncorrected blank values found when p-toluenesulphonic acid was added to only $M/15$ K-SS or Na-SS buffer.

Fig. 35 shows the amounts of carbon dioxide bound by baker's yeast suspended in Na-SS and K-SS buffers in experiments with 50 % oxygen and various proportions of carbon dioxide in the gaseous phase.

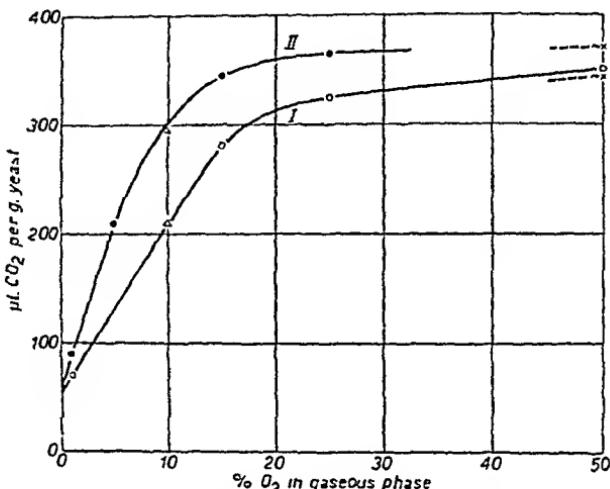


Fig. 36. Bound CO₂ in baker's yeast. Gaseous phase with 25 % CO₂ and varying amounts of O₂.

(I) Suspension in 0.059 M Na-SS buffer.

(II) " " 0.059 M K-SS buffer.

The points X and Δ refer to separate measurements of other yeast samples.

The measurements indicate that the amount of carbon dioxide bound by yeast suspended in K-SS buffer at higher concentrations of carbon dioxide in the gaseous phase is higher than that for yeast suspended in Na-SS buffer. In experiments with a gaseous phase containing 25 % carbon dioxide and different amounts of oxygen, it was observed that, especially at lower concentrations of oxygen, the amount of carbon dioxide bound in yeast in K-SS buffer was considerably greater than when Na-SS buffer was used as suspension medium (Fig. 36).

In an attempt to strengthen the effect of the potassium ions, baker's yeast was shaken in K-SS and Na-SS buffers for 30 minutes at 25° C. in air before the carbon dioxide estimation. No increased effect, however, was found to result from the increase

in the period of action of the potassium ions upon the yeast. In concordance with earlier observations, only smaller amounts of carbon dioxide were bound by both treated yeasts after shaking. In a gaseous phase with, for example, 50 % oxygen + 25 % carbon dioxide + 25 % nitrogen, 315 μ l. carbon dioxide was bound per gram of yeast after treatment with a K-SS buffer, and 280 μ l. in the control with Na-SS buffer.

The increasing influence of potassium ions on carbon dioxide binding was also observed in experiments on budding yeast pretreated in the usual manner, when K-SS buffer was used as suspension medium. The yeast obtained was suspended in Na-SS buffer, and the amount of carbon dioxide bound in the yeast was determined in the presence of 10 % oxygen + 25 % carbon dioxide + 65 % nitrogen in the gaseous phase. In four different experiments, the values 245, 250, 210, and 255 μ l. carbon dioxide per gram of yeast were found. After corresponding pretreatment in a Na-SS buffer, the mean value for the determinations in a gaseous phase of the said composition was only 185 μ l. Thus, after pretreatment of the yeast with glucose + ammonium chloride and K-SS buffer, a much higher amount of carbon dioxide was found when the gaseous phase contained a relatively low concentration of oxygen.

The values of carbon dioxide bound in baker's yeast suspended in Ca-SS buffer and Na-SS buffer collected in Table 26 provide information concerning the influence of calcium ions on carbon dioxide binding. The yeast employed in experiment II bound unusually large amounts of carbon dioxide, as may be seen from the control in Na-SS buffer. From experiments with this yeast and from the two other experiments it results that the calcium ions, in contrast to potassium ions, cause a decrease in the amount of carbon dioxide bound by yeast.

Table 26.

Bound CO₂ in baker's yeast suspended in Na-SS and Ca-SS buffers.

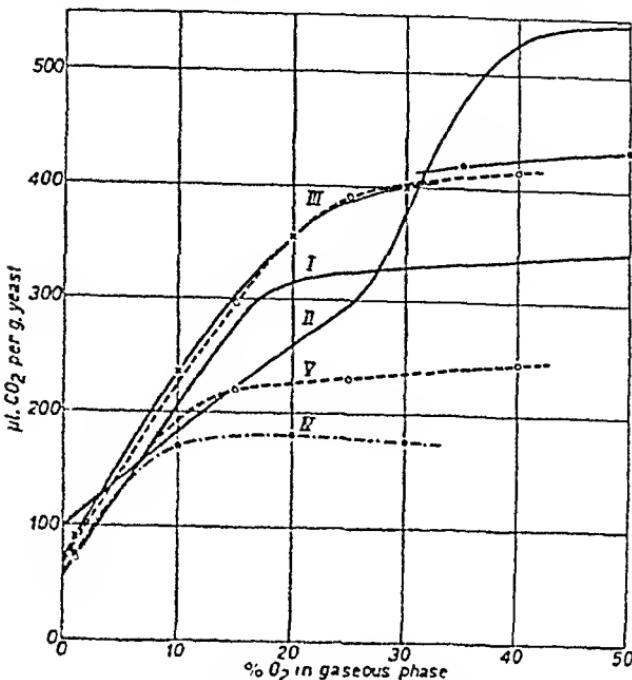
Composition of gaseous phase	μ l. CO ₂ per g. yeast suspended in			
	Na-SS buffer pH 5.14	Ca-SS buffer		
		pH 4.93	pH 5.08	
I 25 % CO ₂ + 10 % O ₂ + 65 % N ₂	230	205	—	
II 25 % CO ₂ + 50 % O ₂ + 25 % N ₂	390	355	—	
III 50 % CO ₂ + 50 % O ₂	490	—	380	

9. Carbon Dioxide in Mother Yeasts.

According to the above observations, the amount of carbon dioxide bound in baker's yeast and in yeasts pretreated in different manners is dependent on the oxygen concentration in the gaseous phase in a manner typical of the individual yeast. In order to decide whether the curves obtained in the investigation of budding yeast were characteristic of growing yeast, experiments were performed both on samples of unripe baker's yeast which, during cultivation in the factory for 11 hours, was in a state of rapid growth, and on samples of vigorously growing mother yeast Z 3 taken after 6 hours cultivation and at the completion of the run. In contrast to the conditions for the propagation of baker's yeast, the growth of Z 3 yeast occurs with considerably weaker aeration. During cultivation, a formation of alcohol therefore takes place, and the yeast assumes a rather anaerobic character. The samples taken from the vat were immediately cooled down to about 5° C. and this temperature was maintained while they were brought to the laboratory. After washing in the centrifuge, the yeast was suspended in water and was filtered on a sintered glass crucible. The yeast thus obtained corresponded in dry weight to baker's yeast. Fig. 37 exhibits the amounts of carbon dioxide bound in samples examined in the presence of 25 % carbon dioxide and different amounts of oxygen in the gaseous phase. For purposes of comparison with the curves for these yeasts, the figure includes the mean curves for baker's yeast (I) and for budding yeast (II).

In Fig. 37, the curves marked III refer to measurements of three different samples of yeast which had been cultivated for 11 hours in the course of baker's yeast production. The measurements indicate that treatment of the samples in the manner described above gives good reproducibility of the values. A comparison of the curves for this yeast and the two samples from the propagation of Z 3 yeast with the normal curves for baker's yeast and budding yeast shows that the course of the curves of the first-mentioned yeast is not equivalent to that of budding yeast, but largely resembles that of baker's yeast. The course of the curve for budding yeast is thus not typical of growing yeasts, but is representative only of the yeast pretreated in the manner described.

This may be a consequence of the different growth conditions in the factory cultivation of the yeast and in the pretreatment

Fig. 37. Bound CO₂ in:

- (I) baker's yeast,
- (II) budding yeast,
- (III) yeast cultivated for 11 hours in preparation of baker's yeast,
- (IV) yeast cultivated for 6 hours in preparation of Z 3 yeast,
- (V) Z 3 yeast.

Gaseous phase with 25 % CO₂ and varying amounts of O₂.

for the preparation of budding yeast. In contrast to the treatment in the preparation of budding yeast, the concentrations of substrate in the industrial process were very small on account of the differential fermentation method used; the temperature, moreover, was somewhat higher. In the first case, the yeast consumed only glucose and ammonium chloride, while in the latter case organic nitrogen compounds, growth substances and numerous inorganic salts were also taken up. The presence of salts during cultivation may, to judge from the experiments described above, be alone sufficient to affect strongly the carbon dioxide binding. It should be emphasized that in the intentionally simplified system yeast-glucose-ammonium chloride a reduced salt content is obtained in the growing cells.

The supposition that the respiration conditions the amount of bound carbon dioxide is lent support by the fact that considerably smaller amounts of bound carbon dioxide are found in the rather anaerobic Z 3 yeasts (IV and V) as compared with the amounts in the aerobic Z 5 yeasts (I and III).

D. Summary of Chapter IV.

WARBURG's manometric method has been modified for the determination of the amount of acid-labile bound carbon dioxide in yeast. In order to obtain a rapid fixation of yeast, different acids were examined: p-toluenesulphonie acid proved to be most suited to this purpose. The vessel constants used for the estimation of the amounts of carbon dioxide expelled from the suspensions were calculated on the basis of the absorption coefficient of carbon dioxide determined experimentally for the mixture of p-toluenesulphonic acid and suspension medium (sodium succinate-succinic acid buffer). Since the solubility of carbon dioxide in the acid or the suspension medium is higher than that in a mixture of these liquids, part of the dissolved carbon dioxide was liberated in experiments with atmospheres containing carbon dioxide. This quantity was determined in the presence of yeast by a special procedure. Possible sources of error are discussed, with special regard to the effect of the yeast metabolism in the determinations.

The amounts of bound carbon dioxide have been determined in different yeasts in equilibrium with gaseous phases containing nitrogen or mixtures of nitrogen and oxygen, with and without carbon dioxide.

In experiments at a constant concentration of oxygen in the gaseous phase, an increase in the bound carbon dioxide was found to accompany an increase in the carbon dioxide content of the gaseous phase. The proportions of carbon dioxide in the gaseous phase ranged between 0 and 50 %.

In experiments with a constant concentration of carbon dioxide in the gaseous phase, it was observed that in all yeasts the amount of bound carbon dioxide increased with increasing concentration of oxygen in the gaseous phase up to a certain limit. In some yeasts, carbon dioxide binding reached a maximum at a given oxygen concentration. A change in the metabolism of the yeast characteristically affected the relationship between carbon dioxide binding and the composition of the gaseous phase (cf. Figs. 32 a and b).

Experiments on baker's yeast made it clear that the carbon dioxide bound by the yeast, when in equilibrium with a gaseous phase rich in carbon dioxide, was liberated again when the yeast was shaken in a carbon dioxide-free atmosphere (Table 23). The amount of carbon dioxide bound in baker's yeast under aerobic

conditions is highest at pH 5.0—5.5 in the suspension medium. The range examined was pH 4—6 (Fig. 26). Carbon dioxide binding in baker's yeast is independent of the illumination.

Experiments have also been carried out with potassium and calcium succinate buffers. The ability of the yeast under aerobic conditions to bind carbon dioxide is markedly affected by these ions. Potassium ions increase the binding, which effect appears most strikingly at relatively low oxygen concentrations in the gaseous phase. The calcium ions, however, reduce the capacity of the yeast for binding carbon dioxide.

The effect of the oxygen concentration on the faculty for binding carbon dioxide is reduced to a high degree by starvation or drying of the yeast. The effect caused by starvation is reversible. A yeast fed after starving is able to bind a many times larger amount of carbon dioxide under aerobic conditions than can starved yeast. Aeration of a suspension of dried yeast overnight imparts a slightly increased power of binding carbon dioxide.

When baker's yeast was treated under aerobic conditions with cell poisons affecting the endogenous respiration (azide, fluoride, cyanide), an effect on the carbon dioxide binding was obtained which ran parallel with the activating or inhibiting effect of the poisons on the respiration of the yeast (Table 25). When the cells were treated with cysteine under aerobic conditions, the capacity for binding carbon dioxide was inhibited.

The augmentative effect of oxygen in the gaseous phase on the power of yeast for binding carbon dioxide is supposed to originate in properties of the protoplasm which depend on the functioning of the respiratory enzymes. This view is supported by the observation that yeast, in which the respiratory enzymes have been injured or blocked by starvation, drying or treatment with cell poisons, is capable of binding only small amounts of carbon dioxide under aerobic conditions. The binding, moreover, is largely uninfluenced by the oxygen concentration in the gaseous phase. Further support for this hypothesis is provided by experiments on mother yeast which acquired a rather anaerobic character during propagation. In this yeast, under aerobic conditions, the fermentation processes dominated over the respiratory processes. Under aerobic conditions, a considerably smaller amount of carbon dioxide was bound by mother yeast thus cultivated than by yeasts grown during vigorous aeration.

CHAPTER V.

pH Determinations and Electrometric Titrations on Heat-fixed Yeasts.

A. Introduction.

We generally defined the acidity in a cell as the average value of the hydrogen ion activity in the water phase in different parts of the cell protoplasm. From numerous investigations on the acidity of different cells it appears clearly that no uniform pH value prevails in the cell (see the reviews by REISS 1926, SMALL 1929, CHAMBERS 1929, SPEK 1937, RIES 1938, and LISON 1941, who discuss the literature from this sphere, the methods and the criticisms offered from various quarters). Observations with the aid of indicators have shown that in one and the same cell the pH values of the hyaloplasm, the nucleus and the vacuoles are divergent (CHAMBERS and POLLACK 1927, CHAMBERS and KERR 1932); furthermore, the pH of the hyaloplasm differs from that of the "granuloplasm" which is clearly differentiated in certain cells (SPEK and CHAMBERS 1933). The pH in the protoplasm, however, does not vary only in the aqueous phase in different parts of the cell. On the surfaces and in their immediate vicinity different cell elements and colloidal particles in the protoplasm presumably have acidities deviating from that in the bulk phase in equilibrium with the surface (SPEK 1937, DANIELLI 1941).

Data concerning the average pH of the intact protoplasm within different cells are extremely divergent, owing to the inaccuracy of the available methods and to the fact that the application of these methods presumably involves an injury to the protoplasm or a change in its characteristics. On the other hand, the pH of the protoplasm is largely dependent upon the physiological state of the material.

The method initially employed for the determination of pH in the cells is based upon the work with indicator dyes. This method is relatively insensitive and uncertain, as the colour of the indicator is affected by numerous disturbing factors, among which may be mentioned the effect of salts, the heterogeneous distribution of the indicator within the protoplasm, the adsorption of the dyes by the plasma colloids, and the precipitation of the dyestuff or its solubility in fatty substances. The alkaline indicators employed previously have proved to cause artefacts and their colours are generally affected by chromotropic substances. The acid sulphophthaleins of CLARK and LUBS have shown to be more appropriate. Since, however, these indicators generally do not permeate the cell membrane, they are used for pH determinations in tissue sections and squeezed or otherwise injured cells, in which materials there is a risk that the initial pH value of the protoplasm has been altered. With the introduction of the micro-injection technique the determinations could be made less violent. Experiments by CHAMBERS and others on sea-urchin eggs, into which indicators had been injected, indicate, however, that even when the egg is pressed or rubbed with a needle, changes in the colour of the indicator appear. These changes are reversible and the indicator rapidly resumes its initial colour.

In recent years, the micro-colorimetric method for pH determinations in cells, introduced by VLÈS (1926), has been improved considerably. By employing monochromatic light and a photoelectric cell, or micro-spectrophotometric measurements on acid indicators with two or more characteristic absorption bands, pH changes in the material may be followed quite objectively (cf. MARGARIA and v. MURALT 1934). FENN and MAURER (1935) emphasize, nevertheless, that in experiments in muscle tissues, the changes in the pH value observed with this method possibly refer to the extracellular pH value, since the dyes of CLARK and LUBS do not penetrate the cells in perceptible amounts.

Numerous investigators have used potentiometric methods for pH determinations, the measurements being performed both on tissue juices and on cells. Just as in the micro-injection of indicators, the introduction of an electrode into the cell involves a great risk of pH changes in the protoplasm. An antimony electrode, for example, was employed by BUYTENDIJK and WOERDEMAN (1927), who measured the pH in amphilbian eggs during their

development. The suitability of this electrode to such experiments was, however, rendered doubtful by the work of DORFMAN (1938), who worked to the same end with a hydrogen electrode. It should be mentioned here that with the latter method the pH values can only be measured under anaerobic conditions, a fact which greatly restricts its applicability.

For pH determinations in tissue juices, quinhydrone electrodes have frequently been employed. FIFE and FRAMPTON (1935) report, however, that measurements on plant juices yielded values differing considerably from those obtained in control measurements with a glass electrode (see also SMALL 1929).

Measurements of pH changes during muscle contraction have recently been performed by MAISON, ORTH and LEMMER (1938) and DUBOISSE (1939), who used a glass electrode and avoided injuries to the cell material. The values obtained refer, however, not to the pH of the muscle cells, but to pH changes found in the tissue fluid between the muscle fibres during the change in carbon dioxide pressure in the cell surface.

Apart from the above methods, pH determinations during muscle contraction have been performed by HILL (1940) by the method of carbon dioxide exchange, using a differential "volumeter" (cf. also LIPMANN and MEYERHOF 1930).

Scarcely any method mentioned above is suited to an accurate estimation of the pH value in the yeast cell. The only determinations performed on yeast, known to the present author, are those by TAIT and FLETCHER (1926) and by MAHDIHASSAN (1930). TAIT and FLETCHER approximately determined the pH in extracts of frozen brewer's yeast, with the aid of indicators, to be 6.2. After the cells had been ground in indicator solutions between the microscope slides, the value 6.0 was found, while, after treatment of the cells in boiling water and centrifugation of the cooled suspension, pH 6.3 was measured in the cell extract. MAHDIHASSAN measured the pH in top yeast by micro-injection of different indicators into the cells. From changes in the colour after injection of acid indicators he estimated the pH to be 5.9—6.0.

In the present investigations, it was of immediate interest to follow possible changes in the acidity of the yeast protoplasm. The only method suited to this purpose seems to be the measurement of the pH in yeast extract or in yeast juice. In view of the resistance of yeast to mechanical treatment, a crushing of, for

example, frozen cells is impracticable within a reasonable time limit. Even if a satisfactory crushing of the cells is attained, the pH determination of the cell pulp involves great risks of a change in its acidity. Even at 0°, the very active decomposing enzymes in the fluid probably cause a shift of the pH value in the acid direction (the so-called postmortal acidification). Not only the proteolytic enzymes, but also nucleases, nucleotidases, dephosphorylating enzymes etc. contribute to a shift in the pH value of the protoplasm.

The use of poisons for the inhibition of enzymic reactions is generally unsatisfactory and their effect on the pH is largely unknown. MEYERHOF and LOHMANN (1926) obtained an inhibition of the glycolysis in muscle extract by means of quinhydrone in pH determinations with a quinhydrone electrode. DANIELSON and HASTINGS (1939) employed ferric fluoride for carbon dioxide determinations in blood, liver and muscle. LEUTHARDT (1940) reported that the addition of sodium fluoride was not effective enough to impede the postmortal acidification in tissue fluid, nor did freezing in liquid air give complete inhibition, since after the preparation was thawed, disturbances were observed as a consequence of incipient enzymic activity.

When cells are fixed by heat, a complete destruction of the cell structures is observed; the contents of, for example, the hyaloplasm and the vacuoles are mixed. Just as in the work on tissue juices, pH determinations on extracts of heat-fixed cells thus yield only values for the average pH in the protoplasm. In comparative pH determinations on the same material under different physiological conditions, measurements on heat-fixed material might provide a good indication of any pH changes occurring.

In the determinations of the acidity in yeast treated under different physiological conditions, the pH was measured in suspensions and extracts of yeasts treated at 100° C. By heat-fixation of dense yeast suspensions, very rapid and effective destruction and inhibition of the enzymic systems in the yeast could be obtained, whereby the postmortal acidification was prevented. Errors involved in the dilution of the cell content with intercellular water can be determined experimentally and pH determinations can be performed at the same concentrations of carbon dioxide in the gaseous phase as that present during the treatment of the cells prior to their fixation.

Beyond an evening out of the pH in different regions of the protoplasm, heat-fixation causes a more or less complete denaturation of the proteins, a process which may affect the pH of the protoplasm. According to BOOTH (1930), heat-denaturation does not change the acid- or base-combining capacity of proteins, despite a change of certain chemical and physical properties in denaturation (cf. ANSON 1938 and the literature cited by him).

With the aid of a hydrogen micro-electrode, LEUTHARDT (1940) measured at a certain carbon dioxide pressure the pH in the surface of thin slices of various tissues which had been heated in a chamber of silver foil to 100° C. in a vapour stream for about $\frac{1}{2}$ minute. He emphasized that after denaturation a change in the titration curve of the proteins, originating from a possible liberation of sulphhydryl groups, could be expected to appear only within a pH range around 8.2 (pK_1' value for the sulphhydryl group in cysteine c. 8.18). In order to check the effect of denaturation on the pH, LEUTHARDT performed pH determinations at different carbon dioxide pressures on native and heat-fixed white of hen's egg and blood serum. He could not detect any essential effect of heat-denaturation on the pH of the material. From this observation he concluded that the tissue proteins in this respect did not behave differently from the egg white and serum investigated.

On the basis of the investigations mentioned above, it was suggested that also in yeast heat-fixation did not essentially affect the average pH of the cell content.

B. Method.

In order to obtain a rapid fixation of the yeast, a thick yeast suspension was squirted through a copper coil ending in an empty conical flask (see Fig. 38). The flask, provided with a short safety tube and a thermometer, was immersed together with the coil in a boiling water bath. The copper coil was 1 m. long with external and internal diameters of 3.2 and 1.5 mm., respectively. For the injection of the yeast suspension a hypodermic syringe of 20 ml. capacity was employed which, without a needle, was directly coupled to a thick-walled rubber tube of small internal radius. The syringe was filled with about 12 ml. suspension and 8 ml. air. During injection of the suspension, the tip of the syringe was held downwards. When the syringe

was emptied completely, the greater part of the suspension present in the coil was expelled by the air in the syringe. It was important that the suspension was removed from the coil, since during prolonged heating the copper reacted with the yeast extract obtained. As receiver was employed a 100 ml. conical flask which was sufficiently large to prevent any foam formed during the development of carbon dioxide from escaping through the safety tube.

When not otherwise stated, in the following experiments 10 g. wet weight of baker's yeast was always used for heat-fixation, being washed directly or after pretreatment with distilled water, centrifuged and diluted with 3 ml. distilled water. Subsequently the suspension was squirted through the heated copper coil. The total time required for the injection of 12 ml. suspension through the coil into the flask was about 6 seconds. The minimum temperature in the flask during and immediately after the injection was 92—93° C. After some seconds, however, the temperature increased again rapidly. A complete heat-inactivation of the cells may be supposed to occur in the course of 2 or 3 seconds. After the injection, the suspension was heated at 100° C. for 5 minutes and immediately cooled down in water to room temperature. For measurements on the extract, the fixed suspension was centrifuged after 15 minutes. Variation in the time of heating (2, 5, 10, and 30 minutes) did not significantly affect the pH of the extract or the suspension.

pH determinations and electrometric titrations were performed on both extracts and fixed suspensions with a glass electrode in conjunction with a valve potentiometer. During measurements at a given carbon dioxide pressure, the solutions were treated with CO₂-containing gas mixtures from gasometers until constant pH was obtained in the solutions. In order to obtain reliable pH values on fixed suspensions, the glass electrode was rinsed repeatedly with water during one and the same pH measurement. Control measurements were subsequently performed on standard pH solutions (phosphate and SS buffers), whereby errors were avoided due to the ready adhesion of the cell material to the

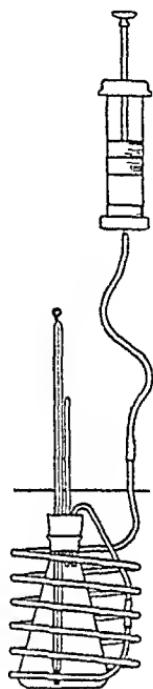


Fig. 38. Arrangement for rapid heating of yeast suspensions.

glass electrode, which caused shifts in the potential corresponding to several hundredths of a pH unit. This, however, was not the case in the work on centrifuged extracts.

C. Experimental Results.

1. Determinations of pH.

The mean pH value in suspensions of 25 samples of baker's yeast, taken at different seasons of the year, was 6.27 ± 0.007 . The mean error of a single determination was 0.034 pH units. When repeated fixations were made of suspensions of the same yeast, a very good reproducibility of the pH of the suspension was obtained (± 0.01 pH units). Preliminary experiments showed that when suspensions of baker's yeast were shaken in air or in a gas mixture containing 50 % oxygen and different amounts of carbon dioxide, the fixed suspensions gave a much higher pH value than that given above. After pretreatment under anaerobic conditions, however, practically the same value was found as in suspensions of untreated baker's yeast. In fed and budding yeasts also the pH was considerably higher than in baker's yeast.

When the fixed suspensions were centrifuged and the pH was determined in the clear solution (the extract), the pH of the extract of baker's yeast thus obtained was practically equal to that found in the suspensions before centrifugation. The extract from yeast, which after pretreatment exhibited higher pH values, regularly showed a value higher by 0.05 pH units than that in the suspensions. This discrepancy between the pH measured in suspensions and that in centrifuged extracts appears most markedly in measurements performed at given concentrations of carbon dioxide in the gaseous phase. Owing to the removal by centrifugation of the solid cellular constituents of the suspensions at a given carbon dioxide concentration, the pH of the extract is considerably lower than that of the suspension.

In order to estimate the pH of the yeast cells at a given concentration of carbon dioxide in the gaseous phase, pH measurements had to be performed at the same carbon dioxide concentration as that prevailing during pretreatment of the yeast. Since the buffering capacity of the centrifuged extract towards carbon dioxide was very different from that of the suspension, pH determinations were performed only of the suspensions and not of the centrifuged extracts in the subsequent experiments.

a) *Dilution Effect.*

Since during heat-fixation of the yeast the cell content is diluted with intercellular and added water, the pH values in the fixed suspensions, measured both in air and in the presence of carbon dioxide, do not correspond fully with those exhibited by the undiluted cell content. This dilution effect on the pH was first examined in experiments on suspensions of baker's yeast diluted with various quantities of water which was added before or after heat-fixation. In the first case, 10 g. of washed yeast was diluted with 2, 3, 5, 10 and 20 ml. distilled water and the suspensions were heat-fixed. In the second case, samples of fixed suspensions of 10 g. of yeast suspended in 3 ml. water were mixed with $\frac{1}{2}$, 1 and 2 volumes of distilled water, the pH being measured in the suspensions thus obtained. In the calculation of the amount of water of dilution, the intercellular water content of the initial baker's yeast was taken to be 25 % of its wet weight (cf. Chapter II, section C 6). After centrifugation the dry weight of the yeast was 22.5 %. Assuming the water content of the cells during washing not to be changed to a notable extent, 10 g. of weighed baker's yeast corresponds approximately to 11.1 g. centrifuged yeast with 2.5 ± 1.1 ml., i.e. 3.6 ml. intercellular water. The sum of this water and the amount added to the suspension as above is denoted as water of dilution. Changes in the pH as functions of the quantity of water of dilution per ml. cellular water are shown in Fig. 39, curves I and II a (cf. Table 27).

As appears from the curves, the pH of the suspensions increases with increasing quantities of water of dilution. When the pH value is extrapolated from curve I to zero water of dilution, i.e. the pH of the undiluted cell content, we arrive at a value lower by 0.07 pH units than that measured in suspensions of 10 g. of yeast in 3 ml. added water (corresponding to 1.32 ml. water of dilution per ml. cellular water). By extrapolation of curve II a, a corresponding change in the pH is found for zero water of dilution, viz. a value lower by 0.06 pH units than that measured in the initial suspension. No essential difference is thus found in the dilution effect in fixed suspensions of 10 g. yeast + 3 ml. water, whether the water of dilution is added before or after heat-fixation. In determinations of the effect of diluting the centrifuged extract of a heat-fixed suspension of 10 g. yeast in 3 ml. water, the same value was obtained.

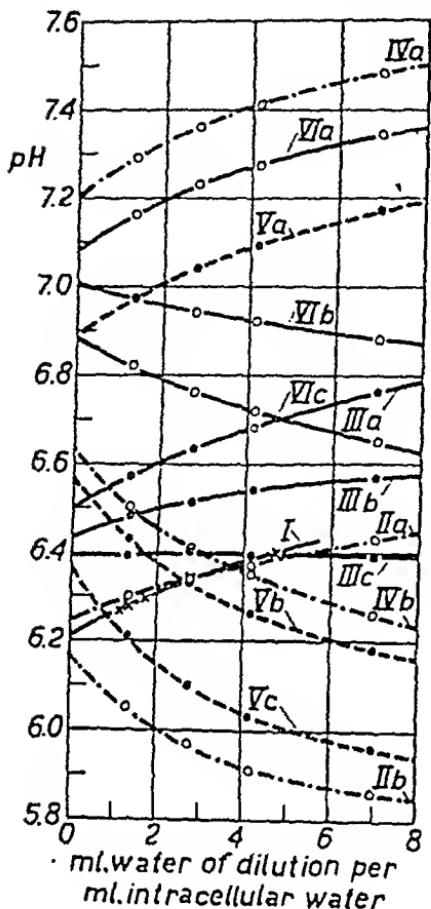


Fig. 39. Effect of dilution on the pH in suspensions of heat-fixed yeasts. I: Suspensions of baker's yeast fixed in the presence of different quantities of water of dilution. pH values measured in suspensions in air. II—VI: Heat-fixed suspensions of 10 g. yeast + 3 ml. water diluted after fixation with different quantities of water. pH values measured in suspensions in air (a) and in equilibrium with gaseous phase containing different amounts of CO_2 (b and c). For the key, see Table 27.

The effect of dilution on the pH was further determined in suspensions of yeasts which after different pretreatments exhibited higher pH values than the heat-fixed suspension of baker's yeast. For practical reasons, the effect of dilution on the pH value was studied only in measurements on dilution series of heat-fixed suspensions of 10 g. yeast + 3 ml. distilled water.

Curves III a, IV a, V a and VI a of Fig. 39 illustrate the dilution effect on the pH in yeast suspensions of various pH values measured in the presence of air. The shape of the curves and the difference between the pH extrapolated to zero water of dilution and that measured at 1.32 ml. water of dilution are in good agreement with those obtained in experiments on untreated baker's yeast (II a). Table 27 shows the difference between the values obtained in heat-fixed suspensions and those evaluated graphically for the undiluted cell content.

The pH values in the heat-fixed suspensions and in samples

Table 27.

Effect of dilution on the pH in heat-fixed suspensions with different pH values of 10 g. yeast + 3 ml. water. The effect determined in air and with gaseous phase containing CO₂ (see Fig. 39).

Yeast	Denota- tion of the curves in Fig. 39	pH deter- mined when gaseous phase contained	Temp. °C.	pH deter- mined in the heat- fixed suspen- sions	pH extrap- olated to zero water of dilution	Dilu- tion effect in pH units
Baker's yeast	I	air	21	6.28	6.21	+ 0.07
Baker's yeast " "	II a	air	21	6.30	6.24	+ 0.06
	II b	25 % CO ₂	21	6.05	6.17	- 0.12
Suspension of baker's yeast ¹ in water, sha- ken for 70 min. at 25° C. in air	III a	air	24	6.57	6.50	+ 0.07
	III b	2.5 % CO ₂	25	6.48	6.44	+ 0.04
	III c	5 % CO ₂	25	6.40	6.40	0
Suspension of baker's yeast in SS buffer, shaken for 40 min. at 25° C. in gaseous phase with 50 % O ₂ + 25 % CO ₂ + 25 % N ₂	IV a	air	24	7.29	7.20	+ 0.09
	IV b	25 % CO ₂	25	6.50	6.63	- 0.13
Suspension of budding yeast in water, sha- ken for 70 min. at 25° C. in gaseous phase with 50 % O ₂ + 50 % CO ₂	V a	air	25	6.97	6.89	+ 0.08
	V b	25 % CO ₂	24	6.43	6.57	- 0.14
	V c	50 % CO ₂	25	6.21	6.36	- 0.15
Fed yeast	VI a	air	25	7.16	7.08	+ 0.08
	VI b	2.5 % CO ₂	25	6.97	7.01	- 0.04
	VI c	5 % CO ₂	25	6.82	6.88	- 0.06

¹ The pH in the heat-fixed suspension of the untreated yeast was extremely low, pH = 6.19.

diluted with water were also determined at equilibrium with a gaseous phase containing carbon dioxide. For 25 % or 50 % carbon dioxide in the gaseous phase the pH value shows an initial rapid decrease with increasing dilution. The pH changes observed in treatment of fixed suspensions with carbon dioxide are reversible, since after heating and aeration of the suspensions the pH value measured prior to carbon dioxide treatment is restored. In suspensions with high pH values, a long period elapses before the carbon dioxide is completely expelled. As shown in

the preceding chapter, the amount of acid-labile bound carbon dioxide in the yeast is largely dependent on the oxygen concentration in the gaseous phase. It was therefore investigated whether the pH of fixed suspensions in the presence of carbon dioxide showed different values in the presence and in the absence of oxygen. In suspensions in equilibrium with a gaseous phase containing 25 % carbon dioxide + 75 % nitrogen or 25 % carbon dioxide + 50 % oxygen + 25 % nitrogen, no difference in the pH could be detected.

When the suspensions were diluted with 1.32 ml. water per ml. cellular water, pH values were obtained, in the presence of 25 % carbon dioxide in the gaseous phase, which were lower by 0.12—0.14 pH units than those graphically evaluated for zero water of dilution. This dilution effect was rather independent of the initial pH value of the suspensions. In the presence of 50 % carbon dioxide, the dilution effect was practically the same (0.15 pH units) (see the examples in Fig. 39, curves II b, IV b, V b, and V c, and Table 27).

Since in the living yeast cell a continuous production of carbon dioxide occurs, a certain carbon dioxide pressure must be supposed to prevail within the cells in suspensions shaken in air. For an estimation of the pH of different yeasts in air it was therefore of interest to know the effect of dilution on the pH of the heat-fixed suspensions at rather low carbon dioxide concentrations such as 2.5 % and 5 % carbon dioxide in the gaseous phase. In contrast to the almost uniform values of the dilution effect in air and 25 % and 50 % carbon dioxide, at low carbon dioxide concentrations different effects are obtained in suspensions with low and high pH values (see Fig. 39, curves III b, III c, and VI b, VI c, and Table 27). In a suspension with an initial pH of 6.57 (III a), in the presence of 5 % carbon dioxide in the gaseous phase (III c), the dilution effect is zero. The increase in pH observed with increasing dilution of suspensions in air is compensated by a tendency for the values to decrease with increasing dilution in the presence of carbon dioxide. In a suspension with an initial pH of 7.16, however, even at 2.5 % carbon dioxide in the gaseous phase the latter effect preponderates (VI a—c). When the initial pH value of the suspension is high, the values measured, even for low carbon dioxide concentrations in the gaseous phase, are thus lower than the extrapolated value for the undiluted cellular content.

b. Estimated pH Values in Baker's Yeast and Yeasts Pretreated under Various Conditions.

As a consequence of the above-described effect of dilution on the pH in heat-fixed suspensions of 10 g. yeast + 3 ml. water, the pH value measured in the presence of different amounts of carbon dioxide in the gaseous phase is lower than that obtained for the undiluted cellular content after correction for the dilution effect. This is especially true for measurements on the centri-

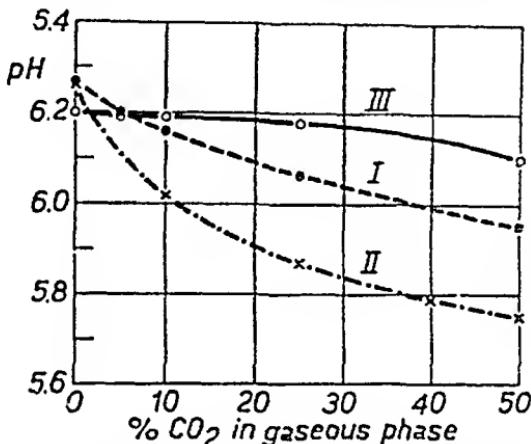


Fig. 40. pH in suspension (I) and centrifuged extract (II) of heat-fixed baker's yeast. Measurements performed in equilibrium with varying amounts of CO₂ in gaseous phase. (III) pH in the undiluted cell content.

fuged extracts of heat-fixed suspensions. Fig. 40 exhibits the pH changes measured in suspensions (I) and centrifuged extracts (II) from baker's yeast. A correction of curve I, on the basis of the dilution effects estimated above for measurements in a gaseous phase with air or with different amounts of carbon dioxide, yields curve III, which then corresponds to the pH changes calculated for the undiluted cellular content of different concentrations of carbon dioxide in the gaseous phase. From curve III it results that the pH in the protoplasm of untreated baker's yeast is almost unaffected by low concentrations of carbon dioxide and that the decrease in pH is relatively small even at high concentrations of carbon dioxide. This phenomenon is of immediate interest, since heat-fixed suspensions of baker's yeast pretreated under anaerobic conditions in the presence of various amounts of carbon dioxide in the gaseous phase have approximately the same pH value as is found in suspensions of untreated baker's yeast when the pH is measured in the presence of air. Curve III

therefore approximately represents the pH values in the protoplasm of baker's yeast under anaerobic conditions at various carbon dioxide concentrations in the gaseous phase.

The pH was further determined in different yeasts pretreated under aerobic and anaerobic conditions both in the presence and absence of carbon dioxide. For this purpose, the yeasts were pretreated in suspensions with the same yeast concentration as was employed in the determinations of the amount of bound carbon dioxide in yeast (Chapter IV). During pretreatment of the different yeasts, 75 ml. yeast suspension, consisting of 10 g. washed yeast in water or in 0.059 M SS buffer, pH 5.1, was shaken in a 450 ml. culture flask provided with a stopper with two stopcocks. In experiments on fed or budding yeasts, only half the amount was used per flask. In this manner, the rather vigorous production of carbon dioxide and consumption of oxygen during the relatively strong endogenous metabolism of these yeasts affected the composition of the gaseous phase to a lower degree.

With constant shaking, 2 litres gas mixture was passed through the flasks over 8—10 minutes. In experiments under anaerobic conditions, the flasks and their contents were rinsed for 2 minutes with a vigorous nitrogen stream prior to the gas passage proper. The suspensions were shaken for 40 or 70 minutes, which period included the time of aeration. The period of 40 minutes corresponds to the time during which the yeast had been shaken in the gas mixture of given composition for the determination of bound carbon dioxide (*i.e.* the period from the beginning of the filling of the WARBURG apparatuses with gas to the tipping of p-toluenesulphonic acid; cf. above). When the shaking was completed, the suspensions were cooled immediately to about +5° C. and centrifuged in ice-cooled centrifuge tubes. When buffer was employed as suspension medium, the yeast was washed once with cooled water. The centrifuged yeast was suspended in 3 ml. water and heat-fixed as described above. The pH was determined in the suspensions in air and in equilibrium with the same carbon dioxide concentration in the gaseous phase as used during pretreatment.

In an attempt to find whether a difference in the pH was obtained after the use of water and Na-SS buffer as suspension media, experiments were performed on baker's yeast which after suspension in water or buffer was pretreated under aerobic or anaerobic conditions in the presence of 25 % carbon dioxide in the

gaseous phase. Repeated experiments did not reveal significant differences between the pH values in yeast pretreated in suspensions in water and in buffer, nor could any effect on the pH be observed when the pretreated cooled yeast had been washed twice instead of once. Since the amount of carbon dioxide bound in yeast under aerobic conditions decreases the more, the longer the yeast is aerated, experiments were undertaken to find whether the pH value of the yeast was reduced by prolonged shaking in a gaseous phase containing oxygen. Pretreatment of the yeast for 40 or 70 minutes under aerobic conditions yielded no significant difference in the pH values, irrespective of whether the yeast had been shaken only in air or in the presence of a mixture of carbon dioxide with 50 % oxygen; the same was the case when the yeast was first shaken for 0—90 minutes in air and subsequently for 40 minutes in a gaseous phase containing 50 % oxygen, 25 % carbon dioxide and 25 % nitrogen.

Table 28 shows the mean values in yeasts pretreated in different manners. The values are corrected for the change in pH described above, which is obtained after dilution of the cellular content by heat-fixation. The standard deviation, calculated from measurements on various yeast samples after different pretreatments, was c. 0.06 pH units. The mean error of the values given in the table, however, amounts in all probability to only about half of this value, since the values given are means in the calculation of which the pH values of the initially untreated yeast are taken into consideration. This could be done, since in experiments on yeast exhibiting initially high and low pH values, a corresponding variation in the pH was obtained after pretreatment. For an estimation of the interdependence between the amount of bound carbon dioxide and the pH in the yeast, reliable values are necessary for the difference between the pH values of yeasts pretreated at similar concentrations of carbon dioxide in the gaseous phase, but under aerobic and anaerobic conditions. In order to reduce the errors originating in biological variations of the yeast, the pH values were as a rule determined simultaneously under aerobic and anaerobic conditions, employing the same initial yeast.

As the pH value of yeast pretreated in a gaseous phase of a certain composition proved to be practically independent of whether the pretreatment had lasted for 40 or 70 minutes and whether the suspension medium was water or Na-SS buffer, all

Table 28.

pH values in yeast pretreated in different manners.

The figures represent means of pH values, corrected for the dilution effect, measured at 23–25° C. in heat-fixed suspensions of 10 g. yeast + 3 ml. distilled water. The values in heavy type represent pH values in yeast at the CO₂-concentration of the pretreatment. The figures in parentheses denote the pH when the intracellular concentrations of CO₂ correspond to 2.5% and 5% CO₂ in the gaseous phase. "40,70" and "water, buffer" denote, respectively, that the relevant values apply irrespective of whether the treatment lasted for 40 or 70 minutes and of whether the medium was water or Na-SS buffer.

No.	Yeast	Time in min.	Suspension medium	Treatment			pH value for cells in equilibrium with gaseous phase containing the following CO ₂ percentage				
				O ₂	CO ₂	N ₂	0 % (air)	2.5 %	5 %	25 %	50 %
1	Baker's yeast	—	—	—	—	—	6.20	(6.19)	(6.19)		
2	„	40, 70	water	20 %	—	80 %	6.63	(6.57)	(6.53)		
3	„	70	water	50 %	—	50 %	6.79	(6.71)	(6.65)		
4	„	70	water	50 %	5 %	45 %	6.96			6.81	
5	„	40, 70	water, buffer	50 %	25 %	25 %	7.18				6.62
6	„	40, 70	water	50 %	50 %	—	7.34				6.47
7	„	40	Na-SS buffer	10 %	25 %	65 %	6.86				6.52
8	„	40	K-SS buffer	10 %	25 %	65 %	6.97				6.56
9	„	70	water	—	—	100 %	6.22	(6.21)	(6.20)		
10	„	70	water, buffer	—	25 %	75 %	6.28				6.25
11	„	70	water	—	50 %	50 %	6.23				6.12
12	Starved yeast	—	—	—	—	—	6.27	(6.25)	(6.24)		
13	„	40, 70	water	50 %	50 %	—	6.47				6.23
14	„	70	water	—	50 %	50 %	6.20				6.10
15	Fed yeast	—	—	—	—	—	7.04	(6.96)	(6.85)		
16	„	40	Na-SS buffer	50 %	25 %	25 %	7.04				6.58
17	„	70	water	50 %	50 %	—	7.05				6.41
18	„	40	Na-SS buffer	—	25 %	75 %	6.71				6.48
19	Budding yeast	—	—	—	—	—	6.89	(6.80)	(6.73)		
20	„	40	Na-SS buffer	50 %	25 %	25 %	6.94				6.55
21	„	70	water	50 %	50 %	—	6.88				6.37
22	„	40	Na-SS buffer	—	25 %	75 %	6.64				6.44
23	Dried yeast un-washed	—	—	—	—	—	6.20				
24	Dried yeast washed once	—	—	—	—	—	6.38				
25	Dried yeast	19 hs	water	20 %	—	80 %	6.39				
26	„	19 hs	Na-SS buffer	20 %	—	80 %	6.26				

pH values listed in Table 28 may be regarded as corresponding to the pH values exhibited by the yeast after treatment with a corresponding gas mixture in the same manner as in the determination of bound carbon dioxide in yeast. The pH values given

or anaerobic conditions refer to experiments in which oxygen was not completely absent. The oxygen concentration in the gaseous phase, however, presumably did not exceed 1 %.

During treatment of yeast suspensions in originally almost CO₂-free gas mixtures, due to the metabolism e. 1—2 % carbon dioxide is obtained in the gaseous phase at the conclusion of shaking. In view of the carbon dioxide production in the cells, the concentration of this gas in the cell may be somewhat higher than would correspond to the composition of the gaseous phase at the end of the treatment, and it presumably increases with the intensity of the endogenous metabolism (cf. p. 157). Especially in experiments with low concentrations of carbon dioxide in the gaseous phase, excessive pH values are obtained if consideration is not paid to the fact that the concentration of carbon dioxide in the cell is higher than that in the gas mixture employed. For an estimation of the pH values in yeasts shaken in originally CO₂-free gas mixtures, Table 28 gives, in brackets, the pH values in the cells calculated under the assumption that a concentration of carbon dioxide is maintained in the cell corresponding to 2.5 or 5 % carbon dioxide in the gaseous phase. In experiments with a gaseous phase containing 25—50 % carbon dioxide, the said increase in the concentration of carbon dioxide in the cell has not been considered.

It is clear from Table 28, Nos. 1—3, that after treatment of a suspension of baker's yeast for 40—70 minutes in air or in a gas mixture of 50 % oxygen + 50 % nitrogen, a considerable increase in the pH of the yeast is obtained, which is more marked at higher concentrations of oxygen. After shaking the yeast for a longer time in air (starved yeast, No. 12), the pH value decreases again. The effect of aerobic pretreatment on the pH in yeast appears most clearly in the presence of carbon dioxide in the gaseous phase. Pretreatment with an addition of only 5 % carbon dioxide to the gaseous phase containing 50 % oxygen causes an increase in the pH value, measured in CO₂-free heat-fixed suspensions, over that found after pretreatment without carbon dioxide (cf. Nos. 3 and 4). After aerobic pretreatment with higher concentrations of carbon dioxide in the gaseous phase, a further increase in the pH value is observed in determinations of the pH in CO₂-free suspensions.

Fig. 41 illustrates the discussed effect of pretreatment on the pH in baker's yeast shaken in a gaseous phase with 50 % oxygen

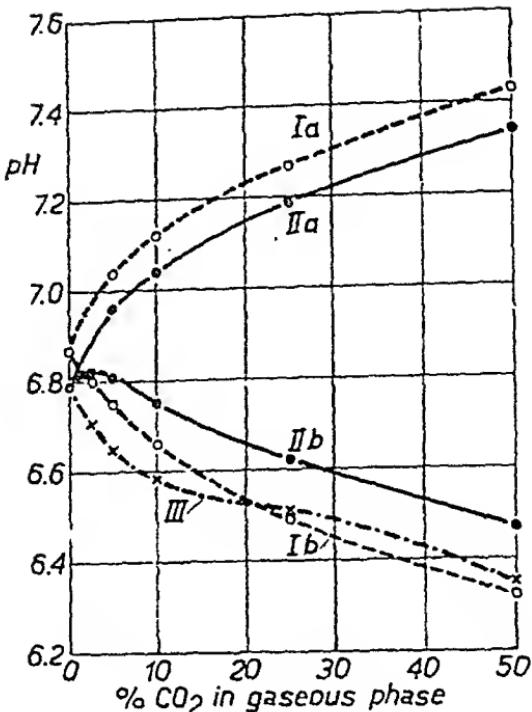


Fig. 41. pH in baker's yeast pretreated for 70 min. at 25° C. in suspension in water with gaseous phase containing 50 % O₂ + varying amounts of CO₂. (pH determinations in heat-fixed suspensions at 23—25° C.).

- (I a) pH in suspension, and (II a) pH in the cells, measured in equilibrium with air.
- (I b) pH in suspension, and (II b) pH in the cells, measured in equilibrium with CO₂-concentration of pretreatment.
- (III) pH in the cells at different CO₂ pressures, if after the pretreatment the values measured in air had been constant.

and 0—50 % carbon dioxide. As examples are given the curves for the pH values *in suspensions* measured both in air and in equilibrium with the same carbon dioxide concentration in the gaseous phase as used in the pretreatments (curves I a and I b). Furthermore, curves II a and II b show corresponding pH values calculated for the *undiluted cellular content*. For purposes of comparison with curve II b, curve III is drawn, which illustrates the pH changes in the cell which might be expected to occur at varying concentrations of carbon dioxide if, after pretreatment, the pH value of the fixed suspensions measured in air did not follow curve I a, but was constant at 6.87. It results from curve II a that the higher the concentration of carbon dioxide is in the gaseous phase during pretreatment, the higher is the pH in the fixed cells measured in air. Despite this, the pH values calculated for the cells in the presence of carbon dioxide (II b) in experiments at

high concentrations of the latter are essentially lower than the value found after pretreatment without carbon dioxide. The pH value of yeast pretreated with 50 % carbon dioxide in the gaseous phase, however, is higher than that in untreated baker's yeast (cf. Table 28, Nos. 1, 4—6).

The increasing effect of aerobic pretreatment on the pH of the cell also appears in experiments with only 10 % oxygen in a gaseous phase containing 25 % carbon dioxide (cf. Nos. 1 and 7 in Table 28). Since the amount of bound carbon dioxide at this oxygen concentration was higher during treatment of yeast suspended in K-SS buffer, pH 5.1, than in Na-SS buffer with the same pH (see Fig. 36), it was investigated whether the pH in yeast shaken in K-SS buffer in the presence of 10 % oxygen and 25 % carbon dioxide was higher than in yeast treated similarly in Na-SS buffer. Nos. 7 and 8 show that this is the case, although the difference in the pH values determined at equilibrium with 25 % carbon dioxide in the cells is quite small.

The marked changes in the pH of the cell under aerobic conditions scarcely occur during treatment under anaerobic conditions. After shaking baker's yeast in a gaseous phase with nitrogen alone or in the presence of up to 50 % carbon dioxide, practically no change in the initial pH value can be observed (cf. Nos. 1 and 9—11 in Table 28 and curve III in Fig. 40). This absence of an effect of pretreatment under anaerobic conditions in the presence of carbon dioxide, which was already mentioned in Chapter III, is also found in the study of titration curves for centrifuged extracts from heat-fixed yeast, as described later.

In agreement with these observations, in experiments on starved yeast performed under aerobic conditions after addition of 50 % carbon dioxide to the gaseous phase, an increase in the pH value measured in air was also observed (Nos. 12 and 13). The pH value of starved yeast under anaerobic conditions in the presence of 50 % carbon dioxide, just as in experiments on baker's yeast (No. 11), is lower than that observed after treatment under aerobic conditions (Nos. 12—14).

The measurements made on baker's yeast and starved yeast show that the changes in the acidity of the protoplasm are intimately associated with a respiration. The pH value found in untreated baker's yeast agrees well with the values for baker's yeast after pretreatment under anaerobic conditions. Untreated baker's yeast may thus be regarded as a yeast pretreated under anaero-

bic conditions. Observations made in experiments on starved yeast indicate, furthermore, that the magnitude of the pH changes in the cell under aerobic conditions is possibly dependent on the magnitude of the endogenous respiration or on the activity of the respiratory enzyme. When baker's yeast is shaken under aerobic conditions in the presence of, for example, 50 % carbon dioxide, a pH value is obtained which is considerably higher than that found in starved yeast under corresponding conditions. In the latter case, the majority of the monomolecular respiration has disappeared and only a weak, rather constant respiration persists.

Observations on the pH in baker's yeast pretreated with sodium azide provided further support for the supposition that respiration plays an essential part in the increase in pH under aerobic conditions. In these experiments, the yeast was pretreated in accordance with the description given in Chapter IV (pretreatment of yeast with different cellular poisons with the aim of determining bound carbon dioxide in the yeast), although with variations necessary for technical reasons. 5 g. yeast was suspended in 300 ml. 0.081 *M* Na-SS buffer, pH 5.1, containing 1.0 millimol. sodium azide per litre. The suspension was shaken in the presence of air for 30 minutes at 25° C. in 1.8 litre culture flasks. It was subsequently cooled, and the yeast was centrifuged and resuspended without washing in 32.5 ml. Na-SS buffer (final concentration 0.059 *M*). This suspension was then shaken in 450 ml. culture flasks in a gaseous phase containing 50 % oxygen + 25 % carbon dioxide + 25 % nitrogen for a further 35 minutes. After cooling, the yeast was centrifuged, washed once and subsequently heat-fixed.

In two experiments in which the yeast had thus been pretreated in the presence of azide, the pH values in the cells measured in the presence of 25 % carbon dioxide were only 6.16 and 6.19, *i.e.* insignificantly lower than the values to be expected for baker's yeast pretreated under anaerobic conditions at this carbon dioxide concentration. This low pH agrees well with the observation that, after pretreatment of baker's yeast at the same azide concentration, the carbon dioxide binding under aerobic conditions is strongly inhibited (Table 25), a finding which, as mentioned in the preceding chapter, may be associated with the marked inhibition of endogenous respiration by azide.

After pretreatment of baker's yeast under aerobic conditions

with glucose or glucose and ammonium chloride, the pH obtained in fed and budding yeasts is higher than that found after shaking a suspension of baker's yeast only under aerobic conditions (Table 28, Nos. 15 and 19). After shaking of both fed and budding yeasts in a gaseous phase containing 50 % oxygen + 25 % or 50 % carbon dioxide, no essential change in the pH value initially measured in air is found (Nos. 15-17 and 19-21). The fact that pretreatment with carbon dioxide under aerobic conditions only slightly affects the pH in the CO₂-free content of these yeasts may be attributed to the intense carbon dioxide production occurring during the initial pretreatment. The carbon dioxide pressure prevailing in the gaseous phase may therefore have been rather high and even somewhat higher within the cells. During the time of the initial pretreatment (2 1/2 hours), the pH value of the cell may possibly have been stabilized. After treatment of the yeast under anaerobic conditions in the presence of 25 % carbon dioxide in the gaseous phase, just as in baker's yeast and starved yeast, lower pH values are obtained in the yeasts than under aerobic conditions (Nos. 18 and 22). The values are, however, considerably higher than the corresponding values for baker's yeast and starved yeast.

pH determinations were also performed on dried yeast pretreated by washing, or shaking suspensions in water or in Na-SS buffer (pH 5.1) under aerobic conditions for 19 hours. The dried yeast employed had been stored in the refrigerator at c. + 5° C. for 19 months. In the preparation of suspensions of unwashed dried yeast for heat-fixation, 2.80 g. yeast was suspended in 11 ml. distilled water. If washed, dried yeast or yeast from suspensions shaken overnight was employed, the centrifuged yeast was suspended in 3.5 ml. water instead of 3 ml. water, as in experiments on other yeasts, since pretreated dried yeast has a lower water content than intact baker's yeast. Table 28 (Nos. 23-26) shows the pH values obtained in dried yeast after various pretreatments. Unwashed dried yeast has a pH of 6.20, increasing after washing to 6.38. As is found from the titrations of extracts from these heat-fixed yeasts described below, the increase in pH after washing originates in a washing of acid components from the yeast. After aeration of the yeast for 19 hours in water, no increase in pH of the protoplasm could be detected. After the yeast was shaken in the presence of Na-SS buffer, pH 5.1, a pH value was found which was even somewhat lower than that

in washed dried yeast. After shaking a suspension of dried yeast overnight, however, a restitution of the buffering capacity of the protoplasm was observed. This is made clear by a comparison of the titration curves for extracts of dried yeasts pretreated in various manners with the corresponding curves for baker's yeast or starved yeast (see below and Fig. 46).

2. Electrometric Titration Curves.

In the preceding sections, only the pH changes occurring in yeast after different pretreatments have been described. For an evaluation of the values observed, the relative buffering capacities in the centrifuged extracts from heat-fixed yeasts were also investigated. Especially in those cases where, after different

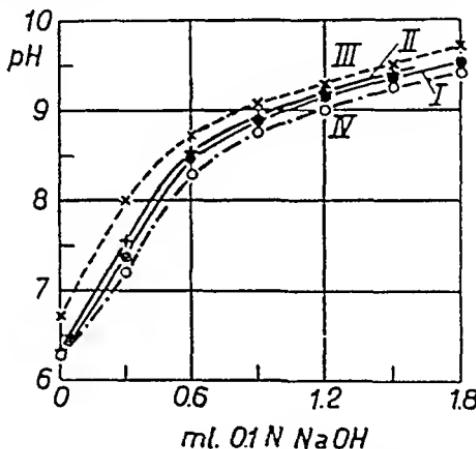


Fig. 42. pH changes during titration of 0.30 ml. centrifuged extract of heat-fixed yeasts.

- (I) Baker's yeast.
- (II) Starved yeast.
- (III) Baker's yeast shaken in air for 70 min.
- (IV) Baker's yeast shaken in N_2 for 70 min.

yeast pretreatments, the pH values were practically unchanged, it should be possible to form some opinion as to whether a difference in the acid- and alkali-binding properties of the cell sap prevails despite the similarity of the pH values. For the study of the buffering capacity, electrometric titrations were performed with sodium hydroxide and hydrochloric acid on 3.00 ml. centrifuged extract from heat-fixed yeast, both being added with a KROGH syringe (0.30 ml.). Since the main part of the insoluble cellular constituents, including the denatured proteins, had been centrifuged off, the titration curves refer principally to the

total amount of the soluble substances of the protoplasm. Figs. 42—46 represent titration curves for yeasts treated in different manners as described above.

Fig. 42 shows curves obtained in the titration of extracts from untreated baker's yeast, starved yeast and baker's yeast pretreated under aerobic and anaerobic conditions. The titration curves exhibit similar courses; that for baker's yeast shaken in

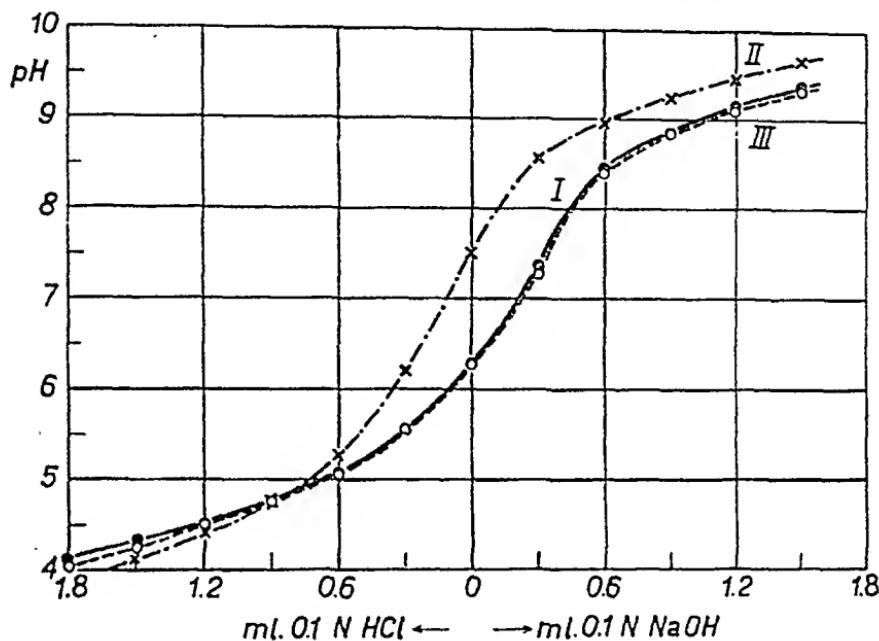


Fig. 43. pH changes during titration of 0.30 ml. centrifuged extract of heat-fixed yeasts.

- (I) Baker's yeast.
- (II) Baker's yeast shaken in gaseous phase containing 50 % O₂ + 50 % CO₂ for 70 min.
- (III) Baker's yeast shaken in gaseous phase containing 50 % N₂ + 50 % CO₂ for 70 min.

air for 70 minutes (III) lies above that for untreated baker's yeast (I). When curve III is displaced along the x-axis, however, it can be brought to coincide completely with curve I. Shaking of baker's yeast in nitrogen or starvation overnight in air does not bring about any important changes, either in the pH of the extracts or in the form of the titration curves. When the yeast is shaken in air for a short time, the pH is increased; since, however, the form of the curve is identical with that of initial yeast, the buffering capacity is practically unchanged. (Only titration with alkali was performed in this case.)

As mentioned previously, after pretreatment of baker's yeast

in an atmosphere containing carbon dioxide but no oxygen, the same pH value was found in the extract as in that of untreated baker's yeast. The courses of the titration curves for these extracts also show good agreement, which appears from Fig. 43 (curves I and III). Pretreatment of the yeast with carbon dioxide in the presence of oxygen, however, causes not only an increase in the pH value of the extract, but also a change in the shape of the titration curves (Fig. 43, curve II).

After pretreatment of starved yeast under aerobic and anaerobic conditions in the presence of carbon dioxide, correspond-

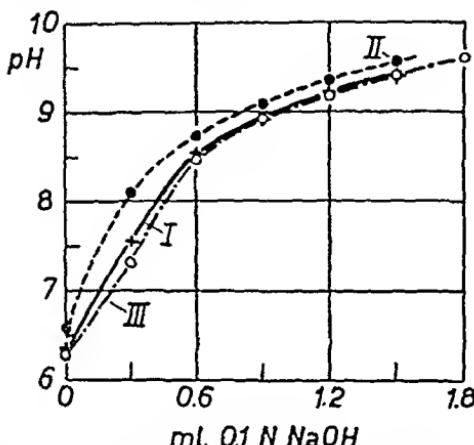


Fig. 44. pH changes during titration of 0.30 ml. centrifuged extract of heat-fixed yeasts.

- (I) Starved yeast.
- (II) Starved yeast shaken in gaseous phase containing 50 % O₂ + 50 % CO₂ for 70 min.
- (III) Starved yeast shaken in gaseous phase containing 50 % N₂ + 50 % CO₂ for 70 min.

ing phenomena were observed (Fig. 44). The divergences between the titration curve of yeast pretreated under aerobic conditions (II) and those of untreated yeast (I) and starved yeast pretreated under anaerobic conditions (III) are but small (cf. corresponding relationships in the reduced respiration and capacity for binding carbon dioxide under aerobic conditions in starved yeast as compared with baker's yeast (Fig. 25)).

In Fig. 45 are given titration curves for extracts from fed (II) and budding (III) yeasts together with those for directly fixed baker's yeast (I). The pH values of the extracts from pretreated yeasts are considerably higher than that from baker's yeast. The course of the titration curves of the pretreated materials deviates from that obtained for the initial baker's yeast.

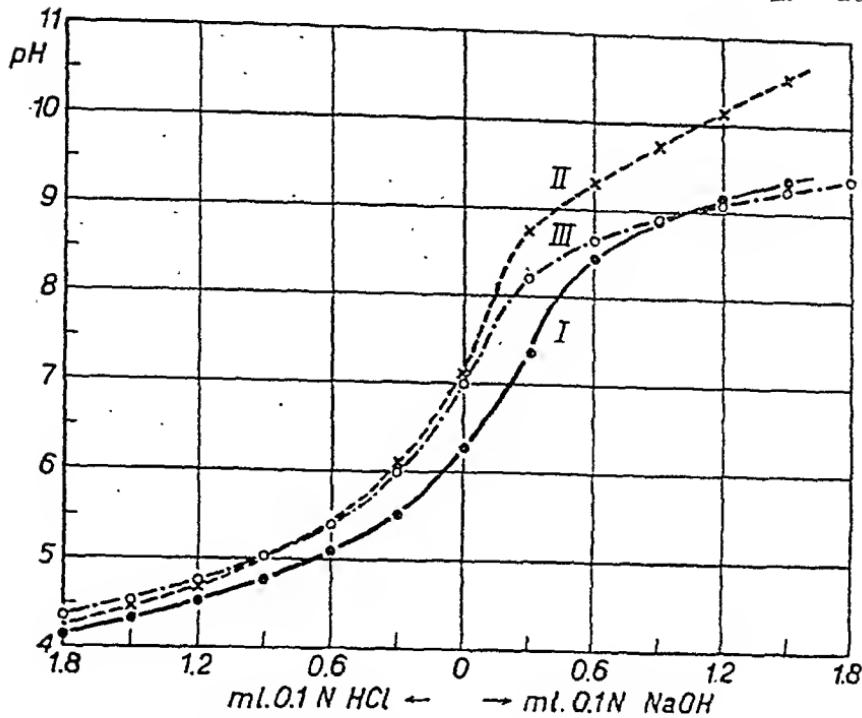


Fig. 45. pH changes during titration of 0.30 ml. centrifuged extract of heat-fixed yeasts.

- (I) Baker's yeast.
- (II) Fed yeast.
- (III) Budding yeast.

A comparison of the curves indicates that curves obtained by titration with hydrochloric acid show practically identical courses. By a displacement of the curves for the pretreated yeasts along the x-axis, the parts of the curves for titration with acid can be made to coincide quite well. However, differences in the courses of the curves are found after titration of the extract with alkali. The extract from fed yeast exhibits an especially decreased buffering capacity against alkali, which indicates a relative reduction in the acid components of the yeast during pretreatment.

Titration curves of extracts from dried yeasts pretreated in the manners described above are collected in Fig. 46. The curve for unwashed dried yeast (II) shows that dried yeast contains relatively great amounts of buffering substances which may possibly be regarded as break-down products formed in the drying and storage of the yeast. It is astonishing that the pH value is not much lower than that of intact baker's yeast; the opposite was to be expected in analogy to a postmortal acidification or a reduction in pH after injury to the cells. If the yeast is washed

only once, a great part of the acid material is removed (cf. curve III). When a suspension of dried yeast is shaken under aerobic conditions in water or buffer, however, titration with sodium hydroxide gives curves (IV and V) which follow very closely the corresponding curve for intact baker's yeast (I), or still better than that of starved yeast. This is in concordance with changes described in Chapter II, Section C, in the permeability of dried yeast and the restitution of its properties by shaking its suspensions under aerobic conditions.

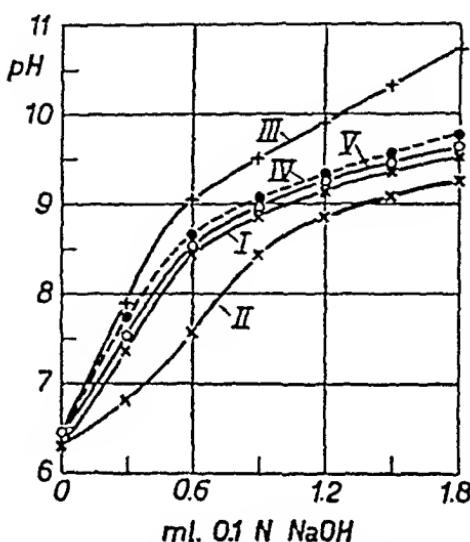


Fig. 46. pH changes during titration of 0.30 ml. centrifuged extract of heat-fixed yeasts.

- (I) Baker's yeast.
- (II) Dried yeast, unwashed.
- (III) Dried yeast, washed once.
- (IV) Dried yeast shaken in Na-SS buffer for 19 hours.
- (V) Dried yeast shaken in water for 19 hours.

Our knowledge of the composition of the yeast plasma, and the changes undergone by its components during alterations in the metabolism, is still too small to serve as a basis for a discussion of the reactions giving rise to the observed changes in pH in the yeast under different conditions. The author will, however, make a few remarks in this connection.

It is tempting to suppose that the large differences in pH in the yeast cell observed under aerobic and anaerobic conditions arise from changes in the acid- and base-binding properties of the protein, when this undergoes variations in its physiological state. As an example of this may be named the difference between

the base-binding capacities of oxidized and reduced haemoglobin. Similarly, it may be imagined that in the transition of yeast from aerobic to anaerobic conditions, or *vice versa*, a change in the quantities of phosphate, nucleic acid, or other compounds bound to the protein would influence its capacity of binding acids and bases.

The observations recounted above of the elevating effect of carbon dioxide under aerobic conditions on the pH value of yeast plasma, measured in fixed CO₂-free suspensions, stand in agreement to the results obtained by THORNTON (1933 b) and FIFE and FRAMPTON (1935) for plant tissues. The finding by FIFE and co-workers (1935, 1941) that, under the influence of carbon dioxide, an increase in the concentration of ammonium ions occurs in the saps of certain plants parallel with the increase in pH, has been found not to apply to yeast (cf. Chapter III, E).

Very little is known regarding the manner in which the phosphate metabolism in yeast cells affects the acidity under different physiological conditions. An esterification of inorganic phosphate usually involves that the phosphoric acid becomes a considerably stronger acid (see values of p_{k₁} and p_{k₂} for ester-bound phosphoric acid collected by ROBISON and MACFARLANE 1940). Under aerobic conditions, the pH in the yeast cell is higher than under anaerobic conditions, both in the presence and absence of carbon dioxide. Analyses show, on the other hand, that the content in the plasma of so-called "free phosphate" under aerobic conditions is very much lower than under anaerobic conditions. The high content of bound phosphate in the former case should, if this were esterified to the intermediate products in the carbohydrate metabolism, rather involve that the pH in the cells was lower than under anaerobic conditions. (In *e. g.* lecithin, however, the bound phosphate is internally neutralized (JUKES 1934).) This conflicts with the experimental findings.

A change in the pH of the yeast cell, however, may be thought equally well to arise from a change in the amount of some acid or in the strength of acidic groups in other compounds, *e. g.* the frequently acidic intermediate products formed in the fermentation processes or entering into the di- and tri-carboxylic acid cycles.

The cellular pH may also be affected by changes in the salt content brought about by diffusion (especially of cations in yeast). Until such time as we know to what extent such processes occur

and in what manner they are conditioned by the metabolism, it appears unlikely that the pH changes in yeast will be satisfactorily elucidated.

By way of conclusion, it may be mentioned that after the subjection of yeast to relatively high concentrations of carbon dioxide under aerobic conditions, the pH of the yeast at the carbon dioxide pressure used in the pretreatment is practically equal to, or somewhat lower than, the pH in yeast treated in the absence of carbon dioxide, but higher than that in yeasts treated under anaerobic conditions either with or without carbon dioxide. Under aerobic conditions, the pH in yeast is thus not radically changed by the presence of carbon dioxide, although such a change was perhaps to be expected, since carbon dioxide clearly affects the metabolism under these circumstances. *On these grounds it appears unreasonable to account for the metabolic effect of carbon dioxide as being due to its direct influence on the mean pH of the yeast cell.*

D. Summary of Chapter V.

A method for pH measurements in yeast has been developed. The pH was determined in suspensions of yeast rapidly heat-fixed at 100° C. The pH values were corrected for the change in pH obtained by diluting the cellular content with intercellular and added water (see Fig. 39 and Table 27).

The pH values in fixed suspensions of yeasts pretreated in different manners were measured both without carbon dioxide and with the same concentration of carbon dioxide in the gaseous phase as employed during pretreatment (Table 28).

The pH value of untreated baker's yeast, which may be regarded as a yeast pretreated under anaerobic conditions, is 6.20.

When a suspension of baker's yeast is shaken *under aerobic conditions*, the pH in the cells is strongly increased. On continued aeration of the yeast (starved yeast), the pH decreases again to almost the same value as prevailed in the initial baker's yeast. During pretreatment of baker's yeast in the presence of carbon dioxide and oxygen in the gaseous phase, the pH further increases in fixed suspensions in equilibrium with air; the pH reduction in the cell occurring in the presence of carbon dioxide is hereby largely counteracted (Fig. 41). The same observations were made in experiments on starved yeast in which, however, the change in pH is considerably less than that in baker's yeast.

Shaking of a suspension of baker's yeast *under anaerobic conditions* brings about no change in the pH of the yeast. In contrast to the treatment under aerobic conditions, pretreatment of the yeast with a gaseous phase containing nitrogen and carbon dioxide causes no essential change in the pH value of CO₂-free heat-fixed suspensions. Thus, in the absence of oxygen in the gaseous phase, and under the sole influence of carbon dioxide, no reactions occur in the yeast to counteract the pH reduction caused by the presence of carbon dioxide (cf. Fig. 40).

On treatment of baker's yeast with sodium azide in a concentration causing an inhibition of the endogenous respiration, the increase in pH found after aerobic pretreatment cannot be observed.

The pH values in fed and budding yeasts were found to be 7.04 and 6.89, respectively. After pretreatment of these yeasts with a gaseous phase containing 50 % oxygen and carbon dioxide, no marked change in the pH measured in CO₂-free heat-fixed suspensions could be detected. The pH value in the cells under anaerobic conditions in the presence of carbon dioxide was considerably higher than in baker's yeast after corresponding treatment, but lower than after treatment of the yeasts with a gaseous phase including oxygen and carbon dioxide.

Electrometric titrations were performed on centrifuged extracts from heat-fixed yeasts. The titration curves show that, after pretreatment of baker's yeast and starved yeast under anaerobic conditions, the presence of carbon dioxide does not influence the course of the titration curves in the yeast extracts.

Unwashed dried yeast has a pH of 6.20. After dried yeast is washed, its pH increases to 6.38. When a suspension of dried yeast is shaken in air for 19 hours, the same pH is obtained as in washed yeast. The titration curves show, however, that the buffering properties of the protoplasm in dried yeast, after aeration, largely corresponded to those of intact baker's yeast; before aeration, the dry yeast had widely different buffering properties.

CHAPTER VI.

Discussion.

A. On the Relation between the Amount of Acid-labile Bound Carbon Dioxide and the pH in the Yeast Cell.

If, in analogy to the conditions prevailing in blood serum, carbon dioxide is primarily bound in yeast to alkali ions, an interdependence should exist between the values found experimentally for the amounts of acid-labile bound carbon dioxide and the corresponding pH values in the protoplasm. Since the pH in yeast is below 7, the formation of carbonate ions is infinitesimal, and only the formation of bicarbonate need be considered.

The relation between the concentration of bicarbonate ions at a certain carbon dioxide concentration and the pH may be expressed by the equation $\frac{[H^+] [HCO_3^-]}{[CO_2]} = k_1$. In this equilibrium equation, k_1 is the first apparent dissociation constant for carbonic acid, which includes the constant for the hydration equilibrium $\frac{[H_2CO_3]}{[CO_2]} = k_o$. $[CO_2]$ in both equations is the molar concentration of carbon dioxide physically solved in water.

Since carbonic acid, H_2CO_3 , is very slightly dissociated in the presence of bicarbonate, $[HCO_3^-]$, coming from $[H_2CO_3]$, can be neglected in relation to $[HCO_3^-]$, which originates from bicarbonate ($= [BHCO_3]$). $[HCO_3^-]$ thus can be put equal to $f \cdot [BHCO_3]$, where f is the activity coefficient for the bicarbonate ions. When $[BHCO_3]$ is substituted for $[HCO_3^-]$, a new equilibrium constant k'_1 is obtained, which includes f . This latter factor may be regarded as being approximately constant in the protoplasm. After the equation is adjusted so as to provide a logarithmic expression for $[H^+]$, the HENDERSON-HASSELBALCH equation is obtained:

$$pH = pK'_1 + \log \frac{[BHCO_3]}{[CO_2]}.$$

According to HENRY's law, the concentration of solved carbon dioxide is proportional to the carbon dioxide concentration in the gaseous phase. BUNSEN's absorption coefficient, α , denotes the number of millilitres of solved carbon dioxide, converted to N. T. P., which are solved in one millilitre of solvent at a given temperature and 760 mm. Hg pressure. According to GUYE and PINTZA (1908), 1 mole of carbon dioxide at 0° C. and 760 mm. Hg occupies a volume of 22.26 litres. If the carbon dioxide pressure is denoted as p_{CO_2} mm. Hg, the concentration of solved carbon dioxide $[CO_2] = \frac{p_{CO_2} \cdot \alpha_{CO_2}}{760 \cdot 22.26}$ moles per litre of solvent. α_{CO_2} for the yeast content is assumed to be constant in experiments with the same yeast both under aerobic and anaerobic conditions. If the experiments are performed at one and the same carbon dioxide concentration in the gaseous phase, $[CO_2]$ is thus constant. If the amount of acid-labile bound carbon dioxide determined experimentally is present as bicarbonate, the difference in the pH value of the cells which should correspond to the difference in the amounts of carbon dioxide bound under aerobic and anaerobic conditions, may thus be calculated in the following manner, irrespective of the values of pK'_i and α_{CO_2} for the yeast content.

$$pH_{aerobic} = pK'_i + \log \frac{[BHCO_3]_{aerobic}}{[CO_2]} \text{ and}$$

$$pH_{anaerobic} = pK'_i + \log \frac{[BHCO_3]_{anaerobic}}{[CO_2]},$$

$$\text{i.e. } pH_{aerobic} - pH_{anaerobic} = \log \frac{[BHCO_3]_{aerobic}}{[BHCO_3]_{anaerobic}}.$$

Since, in this equation, it is immaterial whether $[BHCO_3]$ is given in the absolute concentration per kilogram of water in the cell (cf. below) or in the relative concentration, the values of the previously determined amounts of bound carbon dioxide under aerobic and anaerobic conditions may be inserted immediately, whereupon the following equation is obtained:

$$pH_{aerobic} - pH_{anaerobic} = \log \frac{\mu\text{l. bound CO}_2 \text{ per g. yeast aerobic}}{\mu\text{l. bound CO}_2 \text{ per g. yeast anaerobic}}. \quad (1)$$

The amount of bound carbon dioxide in yeast is several times larger under aerobic than under anaerobic conditions. It has further been observed that carbon dioxide under aerobic condi-

tions greatly affects both the metabolism of yeast and its pH value measured in air, while no effect could be detected under anaerobic conditions. These observations led to the supposition that, under anaerobic conditions, carbon dioxide is mainly bound as bicarbonate; under aerobic conditions, however, it appears to be present also in other forms. If this is the case, in experiments with a given yeast, the difference between the pH values of the yeast in the presence of similar carbon dioxide concentrations in the gaseous phase, with and without oxygen, would be expected to be greater than that found experimentally.

Table 29 shows the measured and the calculated differences in pH for yeasts treated under aerobic and anaerobic conditions, with the same carbon dioxide concentrations in the gaseous phase. The amounts of bound carbon dioxide are corrected for pressure changes in the gaseous phase due to carbon dioxide production by yeast. Since the yeast used for anaerobic pH determinations was presumably not under complete anaerobic conditions, the amount of carbon dioxide bound under anaerobic conditions are taken to correspond to 1 % oxygen in the gaseous phase. As is shown by Table 29, the calculated differences in the pH values of yeast under aerobic and anaerobic conditions are always considerably higher than those found experimentally. This tends to favour the view that part of the acid-labile bound carbon dioxide is not bound as bicarbonate, at least under aerobic conditions.

The discrepancies between the amounts of bound carbon dioxide and the pH values in yeast also appear from the approximate value of α_{CO_2} in yeast calculated below. These calculations have been performed in order to elucidate whether the carbon dioxide can be bound in forms other than bicarbonate under anaerobic conditions also.

In view of the considerable amounts of dry substance in biological material, α_{CO_2} is generally expressed in ml. carbon dioxide per gram of water in the cell and is then denoted as $\alpha_{\text{CO}_2}^{\circ}$. When this definition of α is employed, $[\text{BHCO}_3]$ should be calculated per kilogram of cellular water. In the calculations, it was assumed that 50 % of the weight of fresh baker's yeast consisted of intracellular water, one half of the remainder being dry substance, the other half being intercellular water (cf. p. 73). If carbon dioxide is bound as bicarbonate, we obtain, expressing $[\text{BHCO}_3]$ in moles per kilogram of water,

$$[\text{BHCO}_3] = \frac{\text{ml. bound CO}_2 \text{ in } 2,000 \text{ g. yeast}}{22,260} = \\ = \frac{\mu\text{l. bound CO}_2 \text{ per g. yeast} \cdot 2}{22,260}.$$

Substituting this expression for $[\text{BHCO}_3]$, and the expression for $[\text{CO}_2]$ given above, in the HENDERSON-HASSELBALCH equation, we obtain

$$\text{pH} = \text{pk}' + \log \frac{\mu\text{l. bound CO}_2 \text{ per g. yeast} \cdot 1.52}{\text{p}_{\text{CO}_2} \cdot \alpha_{\text{CO}_2}^{\circ}}.$$

Hence, we obtain

$$\log \alpha_{\text{CO}_2}^{\circ} = \text{pk}' - \text{pH} + \log \frac{\mu\text{l. bound CO}_2 \text{ per g. yeast} \cdot 1.52}{\text{p}_{\text{CO}_2}}. \quad (2)$$

The value for $\alpha_{\text{CO}_2}^{\circ}$ calculated according to equation (2) is only apparent. It is unknown whether the amount of water assumed to be present in the cell is changed during various pretreatments of the yeast and whether the cellular water is fully available as "solvent water" for carbon dioxide. The amount of solvent water in the cells, however, presumably does not vary essentially under aerobic and anaerobic conditions. The values obtained for $\alpha_{\text{CO}_2}^{\circ}$ for the same type of yeast may consequently be assumed to be comparable.

The values of pH, amount of bound carbon dioxide and p_{CO_2} in the above equation can be derived from experimental data. If carbon dioxide bound in yeast occurs only as bicarbonate, the calculated value of $\alpha_{\text{CO}_2}^{\circ}$ should be constant for one and the same yeast under aerobic and anaerobic conditions. There are considered to be no grounds for the supposition that the value of α in the cell is appreciably different in the presence and absence of oxygen. If the bound carbon dioxide under aerobic conditions does not occur completely as bicarbonate, α in the same yeast will be greater when the data found under aerobic conditions are employed than when the calculation is made with data obtained under anaerobic conditions.

The value of pk' calculated with the HENDERSON-HASSELBALCH equation has been determined for serum, haemolysed blood corpuscles and muscle pulp by numerous investigators who have simultaneously studied thoroughly the effect of protein concentration on the pk' value in solutions with given salt contents. CULLEN, KEELER and ROBINSON (1925) found for both human and dog serum $\text{pk}' = 6.095$ at 38° C. and 6.183 at 20°

C. The temperature coefficient was thus — 0.005 per degree C. HASTINGS, SENDROY and VAN SLYKE (1928) found for human serum at 38° C. $pK_1' = 6.11$. Determinations by DILL, DALY and FORBES (1937) yielded the same value for sera from man, ox and dog at 37° C. For cat leg muscle pulp, DANIELSON, CHU and HASTINGS (1939) found a pK_1' of 6.10 at 38° C. They also performed experiments on salt and protein solutions in which, in all cases, the ionic strength of the salts had been adjusted to 0.16 M. The following pK_1' values were obtained at 38° C.: NaCl-NaHCO₃ mixtures with an ionic strength 0.16 gave 6.09, aerated haemolysed ox blood corpuscles 6.09, solutions of hen's egg white or egg albumin with 3—12 % protein and pH 6.38—7.72 gave 6.11. In these experiments, the concentrations were determined in moles per kilogram of water and it was observed that the pK_1' value was not significantly changed in the presence of proteins.

In experiments on haemolysed red blood corpuscles a lower pK_1' value was obtained than in serum, since carbon dioxide in the former system is partly bound to amino groups in the haemoglobin with the formation of carbamate (cf. below). The amount of carbon dioxide thus bound is considerably higher in reduced than in oxidized haemoglobin. The apparent value for the dissociation constant obtained in calculations with the aid of the HENDERSON-HASSELBALCH equation in the presence of haemoglobin, denoted as pK_1^* , is lower, as a consequence of greater carbamate-formation, in reduced blood corpuscles than in aerated cells, being lower in these latter than in serum (see, *inter alia*, VAN SLYKE, HASTINGS, MURRAY and SENDROY 1925, STADIE and HAWES 1928, MARGARIA and GREEN 1933, FERGUSON and ROUGHTON 1934 b, and DILL, DALY and FORBES 1937. The relation between pK_1' and pK_1^* has been treated theoretically by STADIE and O'BRIEN 1937). DILL and co-workers, in experiments on human red blood corpuscles, thus determined $pK_1^* = 5.98$ for reduced, and 6.04 for aerated cells. Similar differences were observed in experiments on ox blood corpuscles. (The corresponding values for ox blood, however, are somewhat higher than those for human red cells; see DANIELSON and co-workers 1939.) In agreement with the measurements on blood serum by CULLEN and co-workers, the temperature coefficient for pK_1^* was found to be — 0.005 per degree C. (determined between 25—37° C.). According to the summary given in LANDOLT-BÖRNSTEIN's phy-

sico-chemical tables, the temperature coefficient for p_{k_1}' is, however, higher (cf. also WARBURG 1922) and may be estimated to be — 0.008 per degree C.

In the determinations of $\alpha_{CO_2}^*$ in different yeasts, the p_{k_1}' value of carbonic acid was assumed to be the same as in muscle pulp or serum. In these materials, the mean for p_{k_1}' at 38° C. is 6.10. Taking the temperature coefficient — 0.008 per degree C., the value of p_{k_1}' was found to be 6.20 at 25° C. As previously mentioned, in view of the carbon dioxide production in yeast, some uncertainty prevails in the evaluation of the carbon dioxide concentration in the cell. The probable errors arising from this concentration become considerably lower, however, when $\alpha_{CO_2}^*$ is calculated from data obtained in experiments with relatively high concentrations of carbon dioxide in the gaseous phase. A correction of p_{CO_2} for the vapour pressure of the suspension medium has not been introduced, since the relatively low vapour pressure at 25° C. can practically be regarded as compensated by the increase in carbon dioxide pressure in the cell due to endogenous metabolism. The values of $\alpha_{CO_2}^*$ calculated for different yeasts, according to equation (2), are given in the last column of Table 29. The calculations show that under anaerobic conditions the value of $\alpha_{CO_2}^*$ becomes considerably lower in all cases than under aerobic conditions, and, allowing for the rather great errors, relatively constant at about 0.5 (with the exception of starved yeast, which reaches only half this value).

A comparison of the values of $\alpha_{CO_2}^*$ calculated for anaerobic conditions in yeast cells with those given in the literature for water, serum or red blood corpuscles reveals that the former values are very low. VAN SLYKE, SENDROY, HASTINGS and NEILL (1928) have determined the solubility of carbon dioxide in human serum and haemolysed ox blood corpuscles at 38° C. In measurements at 1 or $1/2$ atmosphere carbon dioxide pressure, no difference in solubility could be detected. These investigators found that, in the presence of salts, the solubility of carbon dioxide was decreased, while in the presence of lipids, which themselves show a high capacity for binding carbon dioxide, the value of α increased. VAN SLYKE and co-workers report the following values for $\alpha_{CO_2}^*$ at 38° C.: for water 0.547, for serum 0.553, and for haemolysed red blood corpuscles 0.60 ml. carbon dioxide per g. water in the solution. Thus, $\alpha_{CO_2}^*$ in serum and red corpuscles is somewhat higher than in water. At 25° C., $\alpha_{CO_2}^*$ for water is

Table 29.

Values obtained experimentally and calculated from Equation (1) for differences in pH in different yeasts under aerobic and anaerobic conditions at the same CO_2 -concentration in the gaseous phase.

In the last column are given the values, calculated from Equation (2), for $\alpha^{\circ} \text{CO}_2$ for the different yeasts, under the assumption that $\text{pk}'_1 = 6.20$. The pH values are those given in Table 28.

No.	Yeast	Composition of gaseous phase			$\mu\text{l. CO}_2$ per g. yeast	pH	$\text{pH}_{\text{aerobic}} - \text{pH}_{\text{anaerobic}}$			$\alpha^{\circ} \text{CO}_2$
		O ₂	CO ₂	N ₂			Nos.	Found	Calculated	
1	Baker's yeast	50%	25%	25%	340	6.62	1—2	0.37	0.69	1.03
2	" "	—	25%	75%	70	6.25				0.50
3	" "	50%	50%	—	500	6.47	3—4	0.35	0.70	1.07
4	" "	—	50%	50%	100	6.12				0.48
5	" "	10%	25%	65%	210	6.52	5—2	0.27	0.48	0.80
6	" "	10%	25%	65%	300	6.56	6—5 ²	0.04	0.16	1.05
7	Starved yeast	50%	50%	—	145	6.23	7—8	0.13	0.41	0.54
8	" "	—	50%	50%	50	6.10				0.25
9	Fed yeast	50%	25%	25%	325	6.58	9—10	0.10	0.42	1.08
10	" "	—	25%	75%	125	6.48				0.53
11	" "	50%	50%	—	645	6.41				1.59
12	Budding yeast	50%	25%	25%	535	6.55	12—13	0.11	0.73	1.91
13	" "	—	25%	75%	100	6.44				0.46
14	" "	50%	50%	—	645	6.37				1.74

¹ Yeast suspended in K-SS buffer, pH 5.1.

² $\text{pH}_K - \text{pH}_{\text{Na}^+}$.

0.757. If the solubility of carbon dioxide in yeast protoplasm were approximately the same as, for example, in blood corpuscles, the value of $\alpha^{\circ} \text{CO}_2$ at 25° C. should not differ much from the value for water at this temperature, *viz.* 0.76.

For purposes of comparison with the values of $\alpha^{\circ} \text{CO}_2$ given above, it should be mentioned that BROOKS and PACE (1938) have found $\alpha^{\circ} \text{CO}_2$ at 25° C. for fresh hen's egg white to be 0.80 ml. carbon dioxide per g. water in the egg white. DANIELSON, CHU and HASTINGS (1939) have given the following empirical formula for the calculation of $\alpha^{\circ} \text{CO}_2$ in protein-containing solutions:

$$\alpha_t^{\circ} = 0.530 + 0.2 \cdot (\text{protein}) + 0.0125 \cdot (38^{\circ} - t^{\circ}).$$

In this equation, 0.530 is the solubility of carbon dioxide in 0.16 M NaCl at 38° C., expressed in ml. carbon dioxide per g. water. (protein) is the concentration of proteins in g. per g. water. The value of 0.2 was calculated from the data for the effect of

proteins on the solubility of carbon dioxide in acidified serum and in haemolysed blood corpuscles, as reported by VAN SLYKE and co-workers (1928). t° is the temperature in degrees C. By means of the above formula, DANIELSON and co-workers obtained for muscle tissue containing 0.31 g. protein per g. water and with ionic strength 0.16 M , $a_{CO_2}^e$ at 38° C. becomes 0.592 ml. carbon dioxide per g. water in the cells. An estimation of $a_{CO_2}^e$ for yeast at 25° C. with the aid of the same formula, assuming that the ionic strength in yeast cells is also 0.16 and that the protein content in yeast is 0.24 g. per g. cellular water, should yield a value of 0.74.

From these considerations it follows that the value of $a_{CO_2}^e$ calculated for yeast under anaerobic conditions, as given in Table 29, is only two-thirds of the value which might be considered reasonable. The reason for the low value may be found in possible errors in the values of pK'_1 , pH, the amount of acid-labile bound carbon dioxide, and the amount of solvent water in the cell. If it be supposed that all these errors contribute to the low value of $a_{CO_2}^e$, the value of pK'_1 must be too low, pH too high, and the amount of bound carbon dioxide too small. If pK'_1 is higher by 0.05 units, pH lower by 0.05 units, and the amount of bound carbon dioxide higher by 5 % than the values given in Table 29, the calculation with the data obtained in experiments under anaerobic conditions leads to an increase in the value by 32 %, i.e. with the exception of the value for starved yeast, $a_{CO_2}^e$ would become e. 0.65.

Despite the fact that the errors are chosen in such a manner as to co-operate in increasing a , this quantity is still presumably too low, indicating that part of the calculated amount of water in the cell is bound to proteins or other colloids in the protoplasm and is unavailable as "free" solvent water. If this were the case, the amount of bicarbonate per kg. of solvent water in the cell would be higher than that initially calculated and the true value of $a_{CO_2}^e$ should thus be higher than that calculated above. Our knowledge regarding the rôle of bound water is very scanty; it appears probable, however, that the amount of bound water can be rather high, especially in plant tissue and in biological systems containing little water. (A review of the numerous, but frequently contradictory investigations performed with the aim of determining the "bound" water in biological material is given by BROOKS and BROOKS 1941. Cf. also the works by CHANDLER

1941 and FREEMAN 1942.) The low value of α_{CO_2} calculated for anaerobic conditions largely supports this view, simultaneously conforming with the previous supposition that carbon dioxide is only bound as bicarbonate under anaerobic conditions.

The values of α_{CO_2} calculated for starved yeast are considerably lower than the corresponding values for the other yeasts. The cause of this phenomenon is unknown. It appears, however, very probable that a considerable reduction in the water content of the cells occurs during starvation (MALM, private communication), which might partly explain this low value of α .

It results from Table 29 that the values of α_{CO_2} , calculated from data obtained under aerobic conditions are considerably higher than those calculated for anaerobic conditions. This is especially true of the values for fed yeast treated in the presence of 50 % oxygen and 50 % carbon dioxide (No. 11) and budding yeast shaken in the presence of 50 % oxygen + 25 % carbon dioxide (No. 12) or 50 % oxygen + 50 % carbon dioxide (No. 14). The values obtained for the latter yeasts are several times higher than those found for anaerobic conditions. All α values found for yeast under aerobic conditions favour the view that, in the presence of oxygen, a considerable amount of carbon dioxide is bound not only as bicarbonate, but also in other forms. Regarding fed and budding yeasts, the high values of α_{CO_2} are not surprising, since the marked change in the courses of the carbon dioxide binding curves with increasing oxygen concentration in the presence of 50 % carbon dioxide in the gaseous phase (Figs. 29 and 31) suggested that carbon dioxide was not only bound as bicarbonate. The effect of the oxygen concentration on this unknown binding of carbon dioxide is also indicated in the value of α_{CO_2} calculated from data for baker's yeast treated in the presence of 25 % carbon dioxide and only 10 % oxygen in the gaseous phase (No. 5). The calculated value here lies between the values obtained for yeast in the presence of 0 and 50 % oxygen.

Table 29 also gives the measured and the calculated differences between the pH values in baker's yeast suspended in potassium and sodium SS buffers and shaken in the presence of a gaseous phase containing 10 % oxygen + 25 % carbon dioxide + 65 % nitrogen. This difference is denoted by $\text{pH}_K - \text{pH}_{Na}$. Both the experimentally found and the calculated differences in pH are rather small. However, the values of α_{CO_2} for yeasts, both in sodium and potassium buffers, but especially in the latter,

indicate that carbon dioxide is bound not exclusively as bicarbonate in the presence of only 10 % oxygen in the gaseous phase. The effect of potassium ions on the capacity of yeast for binding carbon dioxide can scarcely originate in the small increase in pH of the cells observed in the presence of these ions. The phenomenon should rather be attributed to the enhancing effect of potassium ions on the amount of carbon dioxide bound in an unknown manner under aerobic conditions, an effect which is especially great at lower oxygen concentrations (see Fig. 36).

The calculations thus show that the carbon dioxide bound in yeast under anaerobic conditions probably occurs only as bicarbonate, but that under aerobic conditions carbon dioxide is also bound in another manner. If we consider yeast treated with a constant carbon dioxide concentration in the gaseous phase and calculate, with the help of Equation (1), (on the basis of the determined amounts of carbon dioxide bound under anaerobic conditions and the value of $pH_{\text{aerobic}} - pH_{\text{anaerobic}}$) how great an amount of carbon dioxide is bound as bicarbonate under aerobic conditions (50 % O₂), the following approximate values are obtained. In baker's yeast (in the presence of 25 % and 50 % carbon dioxide), in starved yeast (in the presence of 50 % carbon dioxide) and in fed yeast (in the presence of 25 % carbon dioxide) about half of the total amount of bound carbon dioxide occurs as bicarbonate, while in budding yeast (in the presence of 25 % carbon dioxide) only about one quarter occurs in this form.

B. Possibilities for the Binding of Carbon Dioxide in Yeast in Forms other than Bicarbonate.

A binding of carbon dioxide as bicarbonate may occur within all cells, thus providing a buffering action against pH changes caused by the metabolism. Since the amount of bicarbonate at a given concentration of carbon dioxide is dependent upon the pH value of the cell, the latter will largely determine the rôle of bicarbonate as a buffer, provided that sufficient alkali reserves are available in the cell. From the investigations discussed above, it results, however, that, in contradistinction to the results of observations under anaerobic conditions, carbon dioxide under aerobic conditions can in all probability be bound to a considerable extent in an acid-labile form other than bicarbonate. Carbon dioxide, furthermore, has a pronounced effect on the metabolism

in yeast only under aerobic conditions, simultaneously giving rise to an increase in the pH value of the cell. This parallel between the metabolic effect and the binding of carbon dioxide in forms other than bicarbonate induces us to pay special attention to this form of binding, which may be responsible for the effect of carbon dioxide on yeast.

Several investigators have supposed that a binding of carbon dioxide in acid-labile forms other than bicarbonate occurs in different biological materials. Further details, however, are known only in the case of blood, where carbon dioxide can be bound as carbamate ($R \cdot NH \cdot COO^-$). It is unknown at present to what extent carbon dioxide is bound in an acid-labile form as a pre-stage in the carboxylation reactions which bring about a fixation and incorporation of carbon dioxide in the cell material. The following discussion will therefore be mainly confined to the question as to whether carbon dioxide is also bound as carbamate in yeast. We shall commence with a brief review of existing data regarding the carbamate-binding in blood. It should, however, be emphasized that the part played by carbon dioxide in yeast cannot be regarded as comparable with that in blood, since, from a physiological point of view, these materials exhibit quite different characters. It is obvious that the binding of carbon dioxide in blood both in the form of bicarbonate and in other forms and related changes in pH, ionic exchanges etc., form a special system for the transport of carbon dioxide (and oxygen), while the binding of carbon dioxide in yeast seems to be intimately associated with the cellular metabolism.

A survey of the intensively studied reactions connected with the binding of carbon dioxide in blood has been given by PETERS and VAN SLYKE (1932), who also provided a comprehensive account of the earlier literature from this field. These authors, however, considered only the binding of carbon dioxide in the form of bicarbonate. The view that carbon dioxide could be bound directly to haemoglobin in blood had already been stated by BOHR (1892, 1909). (For earlier literature, see BOHR 1909.) This opinion was supported by BUCKMASTER (1917), MELLANBY and THOMAS (1920), and WARBURG (1922). A strong impulse to the further development of this hypothesis was provided by a series of papers by HENRIQUES (1928 a—e). This author showed that if carbon dioxide were bound as bicarbonate, its rapid liberation in the lung capillaries could not occur, unless the reaction

$H_2CO_3 \rightleftharpoons CO_2 + H_2O$ was catalysed. In the vicinity of the neutral point, this reaction occurs very slowly, a fact observed by VORLÄNDER and STRUBE (1913), THIEL (1913 a and b), and FAURHOLT (1924). If the reaction is not catalysed, carbon dioxide must be bound in forms other than bicarbonate. Judging from observations on the rate of carbon dioxide liberation from haemoglobin solutions *in vacuo* and studies in dialysis equilibria, HENRIQUES supposed the latter alternative to be correct. On the basis of investigations by SIEGFRIED (cf. below) and FAURHOLT (1925), he presented a hypothesis that carbon dioxide was bound to haemoglobin with the formation of carbamate or, according to SIEGFRIED and HOWWJANZ (1909), possibly of carbonic acid esters by reactions with aliphatic hydroxy compounds (see the more detailed discussions by HENRIQUES 1929, 1931). SIEGFRIED (1905) has shown that carbon dioxide is bound to amino acids, peptone, serum albumin, and horse serum. He prepared and studied calcium salts of numerous carbamino acid compounds derived from amino acids and polypeptides (SIEGFRIED 1905, 1906; SIEGFRIED and NEUMANN 1908, and SIEGFRIED and LIEBERMANN 1908). Carbamates from aliphatic primary and secondary amines were later prepared by FICHTER and BECKER (1911).

The different pK' value of carbonic acid obtained by electrometric methods in experiments on serum and haemolysed, reduced, and oxidized blood corpuscles (cf. the preceding section of the present chapter) and investigations by osmotic methods of the same material induced several workers to suppose that carbon dioxide was bound in the red cells not only as free bicarbonate, but also in other forms. Experiments on blood performed by these methods were communicated and discussed in greater detail by ROUANTON (1935), who pointed out the difficulties in drawing any quantitative conclusions from the results, since our knowledge of the effect of proteins on the activities of different ions is very incomplete.

Support for the view that carbon dioxide is bound in forms other than free bicarbonate ions was provided by MARGARIA's (1931 a and b) observations. This author determined the vapour pressure in defibrinated blood and haemoglobin solutions at different carbon dioxide pressures and at 18° C. He showed that the changes in the vapour pressure in solutions in equilibrium with 0.4 % and 10 % carbon dioxide in the gaseous phase amounted only to 60 % of the values to be expected, if it were assumed that

the carbon dioxide taken up in the presence of haemoglobin was osmotically active. This discrepancy increased with increasing concentration of haemoglobin. Experiments on blood serum, however, showed no significative difference between the values found and those calculated for the vapour pressure. MARGARIA therefore supposed that part of the carbon dioxide was bound directly to haemoglobin in an osmotically inactive form.

Further investigations of the binding of carbon dioxide in blood showed that the rapid exchange of carbon dioxide in blood was a catalysed reaction. Simultaneously, carbon dioxide was found to be bound in the blood corpuscles in the form of carbamate. It became clear from the experiments by VAN SLYKE and HAWKINS (1930), BRINKMAN and MARGARIA (1931), and BRINKMAN, MARGARIA, MELDRUM and ROUGHTON (1932) that the rapid uptake and liberation of carbon dioxide in blood must, at least partially, be catalysed. MELDRUM and ROUGHTON (1932, 1933 a) showed that the catalysis was not caused by the haemoglobin; they succeeded in concentrating the enzyme which was named carbonic anhydrase (see also STADIE and O'BRIEN 1933). The enzyme, which is a colourless zinc protein, was later isolated by KEILIN and MANN (1940) and by SCOTT and MENDIVE (1941). Among the numerous data concerning the occurrence of this enzyme, it should be noted that it was found in red cells, but not in serum or in tissue juices. The enzyme could, moreover, be detected neither in yeast nor in green plant material (MELDRUM and ROUGHTON 1933 a). Carbonic anhydrase was, however, found in the blood and the tissues of some marine organisms (BRINKMAN 1933, VAN GOOR 1940).

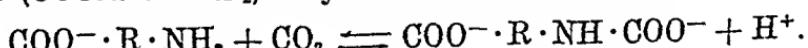
BRINKMAN and MARGARIA (1931) found that the catalytic effect of haemoglobin (or that of carbonic anhydrase) on the liberation of carbon dioxide in bicarbonate solutions was inhibited by potassium cyanide. Continued experiments by MELDRUM and ROUGHTON (1933 b) showed that in blood corpuscles and haemoglobin solutions, despite the inactivation of carbonic anhydrase by cyanide, a more rapid uptake of carbon dioxide was obtained than in pure bicarbonate solutions. This phenomenon was confirmed in control experiments with ammonia, amino acids and peptides. The rapid uptake of carbon dioxide in the presence of cyanide was attributed to the formation of carbamate. In experiments on pure ox haemoglobin, FERGUSON and ROUGHTON (1934 a and b) showed by chemical determinations that carbon dioxide

reacted with this to form a carbamino acid compound behaving in the same manner as the carbon dioxide compounds with amino acids studied by FAURHOLT. In experiments at pH 7.0—7.4 and a carbon dioxide pressure of 30—60 mm. Hg at 38° C., these investigators found that the amount of carbon dioxide bound as carbamate in experiments with oxyhaemoglobin amounted to at the most 2 %, and with reduced haemoglobin to about 10 % of the total amount of bound carbon dioxide.

The discovery that carbon dioxide in the red cells is not only bound as bicarbonate, but also as carbamate, and the discovery of the enzyme carbonic anhydrase which catalyses the hydration and dehydration of carbon dioxide have contributed essentially to the understanding of the conditions prevailing during the transport of carbon dioxide in blood and the effect of oxygen on this transport. Detailed discussions of the reactions involved have been published by ROUGHTON (1935) and by DENOLIN (1938).

Further studies of the formation of carbamate and its reaction equilibria were performed by STADIE and O'BRIEN (1936, 1937) who showed that isolated horse serum proteins also formed small amounts of carbamate with carbon dioxide. These authors emphasized that a formation of carbamate may probably be obtained under suitable conditions with all proteins. The value of the equilibrium constants for the carbamate equilibrium in serum protein, in the presence of carbon dioxide, was determined from the heat of reaction of the carbamate formation. A calculation of the concentration of carbamate in serum at 38° C. and pH 7.4, at a carbon dioxide pressure of 40 mm. Hg, yielded 0.5 ± 0.2 millimoles per litre, an amount which it is difficult to determine exactly.

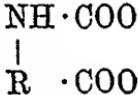
The above-mentioned investigations by SIEGFRIED and co-workers, FAURHOLT, ROUGHTON and co-workers, and by STADIE and O'BRIEN have provided us with considerable knowledge of the properties of carbamate compounds and the conditions necessary for their formation. The formation of carbamate from carbon dioxide and, for example, a monoamino-monocarboxylic acid ($\text{COOH} \cdot \text{R} \cdot \text{NH}_2$) may be written as follows:



Carbon dioxide in the form of CO_2 (not as HCO_3^- , CO_3^{2-} or H_2CO_3) reacts only with the free unionized NH_2 group (this is

also true for the reaction with ammonia and amines). The reaction thus does not take place at the isoelectric point of the amphotolyte, but only on its alkaline side. The carbamate binds one equivalent of base for each mole of bound carbon dioxide. According to FAURHOLT's experiments with amines and amino acids, the percentage part of carbon dioxide chemically bound in the form of carbamate is greatly increased when the pH of the solution is changed from about 8 to 10, although it decreases in more alkaline solutions owing to the reduced free CO_2 -content. Even at the neutral point, small amounts of carbamate may still be formed; the formation of carbamate ceases, however, at slightly lower pH values. The same observations apply to proteins. STADIE and O'BRIEN (1937) have thus shown that carbon dioxide forms, both with oxidized and reduced haemoglobin, small amounts of carbamate at pH 7.0, but not at pH 6.7. These authors showed, furthermore, that carbamates formed according to the above equation are present as salts of dibasic acids at all pH values above 7.

The resulting carbamates form soluble calcium and barium salts such as $\text{NH}\cdot\text{COO} \text{---} \text{Ca}$, which, in alkaline solution, may be



employed for the differentiation of carbamates from carbonates. The salts are rather stable at 0° C. and pH c. 12 or higher, under which conditions their dissociation rate is low, although they split off carbon dioxide immediately in acid media. Since the heat of formation of carbamate is very high, the reaction is favoured by low temperatures. Experiments at 70° C. have shown that the bound carbon dioxide is rapidly split off at this temperature.

In yeast, the amount of carbon dioxide bound in forms other than bicarbonate and the conditions essential for its binding as carbamate are quite different from those in blood. In the paper of FERGUSON and ROUGHTON (1934 b), carbon dioxide bound in forms other than free bicarbonate ions is denoted as *x-bound* CO_2 . According to the estimations in the preceding section of this chapter, approximately half of the carbon dioxide bound in different yeasts with a gaseous phase containing 50 % oxygen + 25—50 % carbon dioxide consists of x-bound CO_2 . In budding yeast, in the presence of 25 % carbon dioxide in the gaseous phase, up to about three-quarters of the bound carbon dioxide is estimated to be x-bound. In all cases, the pH value in the yeast

is about 6.6, apart from starved yeast, in which the value is only about 6.2. In view of the above considerations regarding the dependence of carbamate-formation on the pH, the formation of carbamate in yeast at the said pH values is in all probability zero or extremely small.

The observation that the quotient $\frac{\text{bicarbonate-CO}_2}{\text{x-bound CO}_2}$ in yeast

is about 1 is evidence for the view that at least the main part of the x-bound CO₂ is not carbamate. Experimental data in the literature concerning the formation of carbamate in the presence of bicarbonate indicate that this quotient is considerably lower than unity at different carbon dioxide pressures. At higher carbon dioxide pressures, the quotient decreases to the order of magnitude of 0.1 and still lower, despite the fact that the experiments were performed at a pH which is more favourable to the formation of carbamate than is the pH in yeast. The formation of carbamate with, for example, mammalian haemoglobin is greatest at very low carbon dioxide pressure, decreasing rapidly with increases in this pressure. A comparison of the amounts of x-bound CO₂ in yeast and in serum strongly supports the view that carbon dioxide in yeast is not bound as carbamate. The concentration of the x-bound CO₂ in, for example, baker's yeast in a gaseous phase with 50 % oxygen + 50 % carbon dioxide may be estimated at 20 millimol. per kg. of cellular water. Although x-bound CO₂ is found in yeast at a low pH value, the amount of x-bound CO₂ is about 40 times higher than the calculated amount of carbamate in serum at pH 7.4, 40 mm. carbon dioxide pressure and 38° C. (see above). Even compared to the amount of carbamate formed in red blood cells with reduced haemoglobin, which has a high capacity for binding carbon dioxide in this manner, the amount of x-bound CO₂ in yeast is several times greater (see STADIE and O'BRIEN 1937).

To judge from the above data, it appears, however, not impossible that a minor part of carbon dioxide in yeast may be bound as carbamate. Especially at lower carbon dioxide pressures, under aerobic conditions, when the pH value in yeast is only somewhat below 7 and that within certain cell structures is possibly still somewhat higher, the conditions for the formation of carbamate may be somewhat more favourable than at higher carbon dioxide pressures.

Finally, it should be mentioned that haemoglobin at reduced oxygen pressure binds an increased amount of carbon dioxide as carbamate, while in yeast the amount of x-bound CO_2 increases with increasing concentration of oxygen in the gaseous phase. This contrast, however, may be caused by the essential physiological differences between the two materials and cannot be taken as conclusive evidence that no formation of carbamate takes place in yeast.

It results from this discussion that x-bound CO_2 in yeast does not consist of carbamate, at least in the greater part. The residual quantity, which is bound neither as free bicarbonate ions nor as carbamate, is denoted by FERGUSON and ROUGHTON (1934 b) as *y-bound CO_2* (equal to x-bound CO_2 — carbamate-bound CO_2). This denotation will be adopted here. The occurrence of such an unknown binding of carbon dioxide in blood was deduced by FERGUSON and ROUGHTON from discrepancies obtained in the calculation of pK_1' for carbonic acid by means of the HENDERSON-HASSELBALCH equation, despite their taking into consideration the analytical values for carbamate (see discussion by ROUGHTON 1935).

Further support for the view that carbon dioxide can be *y-bound* was provided by the investigations of WILLMER (1934) who observed that the blood of certain tropical fresh-water fishes from Guiana showed a very low pH value (the types investigated usually had about pH 6.7). In two species, *viz.* *Pterodoras granulosus* (VAL.) and *Hoplias malabaricus* (BLOCH), the pH of the blood was only about 6.0. From the few determinations performed, it was clear that in the blood of these fishes considerably more carbon dioxide was bound than could be accounted for by the HENDERSON-HASSELBALCH equation. A formation of carbamate at the pH value in question is very improbable.

Experiments by FERGUSON, HORVATH and PAPPENHEIMER (1938) concerning the transport of carbon dioxide in dogfish blood (*Mustelus canis* and *Squalus acanthias*) indicated that the blood of these fishes binds carbon dioxide in forms other than bicarbonate or carbamate. The binding of carbon dioxide in dogfish blood was independent of whether the haemoglobin was oxidized or reduced. The total concentration of bound carbon dioxide per unit weight of water in the red cells was higher than in the serum in equilibrium with the blood corpuscles; this circumstance stands in contrast to the corresponding relationships in mammalian blood.

An approximate calculation of the excess of carbon dioxide in the red cells showed that this amount increased with increasing carbon dioxide pressure, which fact supported the view that the bound carbon dioxide did not occur as carbamate. The authors supposed that the increased carbon dioxide binding in the red cells was due either to a binding of bicarbonate ions to protein or to the nuclei in the blood corpuscles being more alkaline than the surrounding cytoplasm (cf. CHAMBERS and POLLACK 1927), which latter fact might contribute to an increased binding of carbon dioxide as bicarbonate.

Investigations of the state of bound carbon dioxide in mammalian muscle have recently been communicated by CONWAY and FEARON (1944), who suppose that a great part of the carbon dioxide in the muscle is not bound as bicarbonate, but in another manner. The carbon dioxide was extracted from the muscles with alkali hydroxide and the carbon dioxide liberated after acidification of the extract was determined. The values obtained agree well with those found by WALLACE and HASTINGS (1942) (see also DANIELSON and HASTINGS 1939). By adding barium chloride to the alkaline extract and centrifuging off the resulting precipitate, CONWAY and FEARON succeeded in showing that about half of the acid-labile bound carbon dioxide was not precipitated by the barium ions. If, before adding barium chloride, bicarbonate was added to the alkaline extract, this latter was precipitated completely. On the basis of determinations of the rate at which carbon dioxide was given off *in vacuo* from guinea pig muscle preparations, the authors supposed that somewhat less than half of the soluble barium salts corresponded to carbamate-bound carbon dioxide, the remainder being bound in an unknown form.

The occurrence of carbamate-bound carbon dioxide, however, appears improbable at the low pH value calculated by CONWAY and FEARON for the muscle cell on the basis of DONNAN equilibria, *viz.* pH 6.0. The pH in the muscle cell is presumably considerably higher, *i. e.* only slightly below 7 (see FENN and MAURER 1935). In their brief paper no account is given of the method used. It appears probable, however, that part of the carbamate-bound carbon dioxide observed in the muscle might have been formed secondarily in the alkaline muscle extract. According to FERGUSON and ROUGHTON (1934 b), a secondary carbamate formation is difficult to eliminate without using a special method, when a solution containing, for example, protein is made alkaline

in the presence of carbon dioxide. CONWAY and FEARON's observations that a γ -bound CO_2 fraction might be present in the muscle cells are, however, concordant with the observations mentioned above on the blood of certain fishes and with the present author's experiments on yeast.

The above considerations show that the occurrence of carbon dioxide in γ -bound form has been established by several investigators; more detailed data concerning the nature of this binding are, however, completely lacking. Carbon dioxide or bicarbonate ions may perhaps enter into reversible combination with protein to form an undissociated or slightly dissociated compound, in accordance with the theory presented from several quarters that cations, under physiological conditions, can also reversibly combine with proteins through a more or less dissociated linkage. Convincing experimental support for this theory has not yet been provided. If carbon dioxide were bound to protein, the difference observed between the capacities of yeast for binding carbon dioxide under aerobic and anaerobic conditions should represent certain changes in the state of the yeast proteins, which should accordingly be different under aerobic and anaerobic conditions.

According to SIEGFRIED and HOWWJANZ (1909), carbon dioxide in a moderately strong alkaline medium may combine with aliphatic polyhydroxy compounds with the formation of carbonic acid esters. BAUER and NAMEK (1940), who investigated the formation of esters from aliphatic alcohols and carbon dioxide, have shown that this reaction is very slow, although it occurs both in neutral and in weakly acid solutions. It seems by no means excluded that in yeast, which contains considerable amounts of carbohydrates and derivatives thereof, a rapid formation of carbonic acid esters may occur under physiological conditions with the aid of some enzyme. Undoubtedly, certain striking analogies here present themselves with the formation of phosphoric acid esters. This latter is favoured by aerobic oxido-reduction processes, as is the binding of carbon dioxide in the γ -bound form.

Finally, it should be mentioned that carbon dioxide can be fixed enzymically to pyruvic acid with the formation of oxaloacetic acid or its derivatives, a reaction which may be supposed to occur also in yeast (Chapter I). As oxaloacetic acid in strongly acid solution gives off the bound carbon dioxide, it appears possible that a certain amount of the acid-labile bound carbon dioxide in yeast originates from oxaloacetic acid or from a compound in

which carbon dioxide is bound in a similar manner. In order to permit a decision whether such a form of binding may occur to a considerable extent in yeast, more detailed data are necessary concerning the equilibria in carboxylation reactions and the reactions of the products formed under different physiological conditions. It should be mentioned that KLEINZELLER (1941), in experiments on yeast, has found that approximately equal amounts of succinic acid are formed under both aerobic and anaerobic conditions. If we assume that succinic acid is formed by way of oxaloacetic acid, these observations indicate that carboxylation reactions in yeast are independent of the oxygen pressure. If γ -bound CO_2 in yeast originates from oxaloacetic acid, the amounts of such carbon dioxide would be expected to be equal under aerobic and anaerobic conditions. The amount of γ -bound CO_2 in yeast is, however, highly dependent on the concentration of oxygen in the gaseous phase, which fact discounts the view that it originates from oxaloacetic acid.

General Summary.

The experimental results of this investigation have been summarized at the ends of Chapters II—V (pp. 73, 89, 136 and 164). The reader is therefore referred to the separate summaries for more detailed information.

The object of the present work was to study the effect of carbon dioxide upon the metabolism of yeast and the changes here brought about in certain of the components of the plasma, in the power of the cells to bind carbon dioxide in the acid-labile form, and in the acidity of the cells. Only a few preliminary investigations in this sphere have previously been carried out on yeast. The action of carbon dioxide on the living cell is therefore only known from diverse researches performed on other biological materials. A summary of these is given in Chapter I.

Baker's yeast was employed as the experimental material. By its pretreatment by starvation, by feeding with glucose in the presence and absence of ammonium chloride, and by drying, different yeasts were obtained which showed metabolisms differing characteristically in intensity and quality from that of the untreated yeast. A few experiments were carried out on mother yeasts, originating in the industrial production of baker's yeast. The yeasts were generally suspended in succinic acid—sodium succinate buffer of pH 5.1. All experiments were performed at 25° C.

The effect of pretreatment on baker's yeast was investigated by studying the cytology, endogenous respiration and fermentation of the different materials. Determinations were also made of the turnovers of carbohydrates and nitrogen and phosphorus compounds in yeasts during pretreatment together with the changes in the dry weights of the products. A review of the modes of preparation of the yeasts and a description of their properties are given in Chapter II, pp. 47 and 73.

An investigation was made of the manner in which the charac-

ter of the metabolism affects the capacity of the yeasts of binding carbon dioxide and the acidity of the cells. Experiments were made with different concentrations of carbon dioxide in the gaseous phase under aerobic and anaerobic conditions.

Chapter III deals with a study in the effect of the presence of 15—50 % carbon dioxide in the gaseous phase on the growth, metabolism and cell components of the yeasts. These experiments were chiefly performed on baker's yeast.

Experiments under *aerobic* conditions showed that carbon dioxide has a distinctly inhibitory effect upon the growth of yeast (Table 7), but activates the endogenous respiration (Table 8) and the uptake of glucose (Figs. 17 and 18) and pyruvic acid (Table 11). The cell content of free phosphate diminishes when carbon dioxide is present in the gaseous phase, but this diminution is not brought about by a release of phosphate ions into the suspension medium (Tables 9 and 10). The very low ammonium ion concentration of the protoplasm is unaffected by carbon dioxide.

Under *anaerobic* conditions, however, carbon dioxide has no influence on the cell metabolism, for the velocities of uptake and fermentation of glucose are found to remain unchanged, irrespective of whether carbon dioxide is present (Fig. 19). The free phosphate content of the cells also remains unchanged during treatment with carbon dioxide under anaerobic conditions (with the exception of yeast which has begun to grow during pretreatment; see Table 10).

Chapter IV includes an account of estimations of the amounts of acid-labile bound carbon dioxide in different yeasts in equilibrium with gaseous phases containing different proportions of oxygen, nitrogen and carbon dioxide. A modification of the WARBURG procedure was worked out for these estimations. The yeast was fixed for this purpose with *p*-toluenesulphonic acid. Determinations of the absorption coefficients of carbon dioxide (α) in the sulphonic acid and the suspension medium showed that α was greater in these liquids individually than in the mixture of them obtained in the fixation (Table 14). The amount of dissolved carbon dioxide consequently liberated in the presence of carbon dioxide in the gaseous phase was determined with regard to the effect on α of the fixed yeast content.

Determinations of the amounts of acid-labile bound carbon dioxide in different yeasts showed that changes in the yeast

metabolism modified in characteristic manners the dependence of the binding of carbon dioxide on the composition of the gaseous phase.

Experiments with a *constant oxygen concentration* in the gaseous phase showed, for all yeasts investigated, that the amount of bound carbon dioxide increased with the carbon dioxide content in the gaseous phase. The concentration range studied was 0—50 % carbon dioxide (Fig. 32 a).

Experiments with a *constant carbon dioxide concentration* in the gaseous phase indicated that, in all the yeasts, the amount of carbon dioxide bound increased, to a certain limiting value, with the oxygen concentration in the gaseous phase. In certain yeasts an optimum binding of carbon dioxide was found at a certain oxygen concentration (Fig. 32 b).

Experiments with baker's yeast showed that the aerobic binding of carbon dioxide was reversible and attained its maximum at pH 5.0—5.5 in the suspension medium. It was not affected by irradiation. Potassium and calcium ions, in the presence of oxygen, had increasing and decreasing effects respectively on the amounts of carbon dioxide bound.

The greatly increased capacity of yeast, in the presence of oxygen, of binding carbon dioxide led to the assumption that this capacity was associated with properties of the protoplasm conditioned by the occurrence of a respiration and partly by the intensity thereof. Support for this hypothesis was provided by the results of experiments with baker's yeast in which the respiratory enzymes were damaged or blocked by starvation, drying, or treatment with respiration-inhibiting cell-poisons (NaN_3 , NaF , KCN or cysteine). In all cases where its respiration was inhibited, the yeast could bind, under aerobic conditions, only a fraction of the quantity of carbon dioxide bound by untreated baker's yeast. This fraction was affected only slightly by the oxygen concentration in the gaseous phase. On the other hand, in yeast where the respiration had been activated by low concentrations of cell-poisons, an increased power of binding carbon dioxide was observed (Table 25).

In order to investigate whether the difference between the capacities of the yeast of binding carbon dioxide under aerobic and anaerobic conditions was due to a difference in the pH in the cell, the pH was measured in different yeasts treated in gaseous phases of different compositions (Chapter V). In the procedure

worked out for measuring the pH in the yeast, the suspensions were fixed by very rapid heating to 100° C. The pH was measured in the fixed suspensions and centrifuged extracts in equilibrium with air and a gaseous phase with the carbon dioxide concentration prevailing during the pretreatment. The values were corrected for the effect on the pH of a dilution of the cell content (Fig. 39 and Table 27).

The determinations showed that the pH in all yeasts, in atmospheres free from or containing carbon dioxide, was lower than under the corresponding aerobic conditions.

Treatment of baker's yeast with a gaseous phase containing carbon dioxide under *anaerobic* conditions brings about no appreciable variation in the pH of the CO₂-free heat-fixed suspensions. The reduction in pH caused by the presence of carbon dioxide in the cells (Fig. 40) is thus not counteracted by reactions in the protoplasm under the influence of carbon dioxide.

Under *aerobic* conditions, however, after pretreatment of the yeast in the presence of carbon dioxide, a higher pH is found in fixed suspensions in equilibrium with air than is measured in suspensions pretreated in the absence of carbon dioxide. The reduction in pH occurring in a cell in the presence of carbon dioxide is thus counteracted under aerobic conditions by reactions involving a displacement of the pH towards the alkaline side (Fig. 41). Further data regarding the pH values of yeasts treated in various ways are given in Table 28 (see also summary to Chapter V, p. 164).

A calculation was performed of the differences between the pH values in yeasts under aerobic and anaerobic conditions for constant carbon dioxide concentration in the gaseous phase. The pH differences calculated on the basis of the quantities of carbon dioxide found analytically were considerably greater than those found experimentally (Table 29). The apparent value of the absorption coefficient of carbon dioxide (α^o) for the cell content was found from the experimental data for the pH and the amount of carbon dioxide bound (Table 29). For yeast under *anaerobic* conditions a relatively low value of α^o was obtained. Under *aerobic* conditions, however, a higher value was always found, amounting in certain cases to 2—3 times the value for anaerobically treated yeast. A discussion of these calculations has led to the conclusion that the carbon dioxide bound in an acid-labile form in yeast under *anaerobic* conditions most probably occurs only as bicar-

bonate, while under *aerobic* conditions carbon dioxide is largely bound in another manner.

The observation that carbon dioxide influences the metabolism of yeast only under aerobic conditions, and that it is bound in a form other than bicarbonate only under these conditions, gives cause to focus attention upon this mode of binding, which is possibly associated with the metabolic effect of carbon dioxide. The nature of the binding is not known, although several possibilities are discussed. It is concluded that only a small part of the carbon dioxide can be bound as carbamate.

References.

ADAIR, G. S. and ADAIR, M. E. (1934), The determination of the membrane potentials of protein solutions and the valence of protein ions. *Biochem. J.* 28, 199.

ADOLF, M. und PAULI, W. (1924), Untersuchungen an elektrolytfreien, wasserlöslichen Proteinkörpern. II. Beziehungen zu Kohlensäure. *Biochem. Z.* 152, 360.

ANNAU, E., BANGA, J., GöZSY, B., HUSZÁK, ST., LAKI, K., STRAUB, B. und SZENT-GYÖRGYI, A. (1935), Über die Bedeutung der Fumarsäure für die tierische Gewebsatmung. *Hoppe-Seyl. Z.* 236, 1.

ANSON, M. L. (1938), The coagulation of proteins. In SCHMIDT, C. L. A., The chemistry of the amino acids and proteins. Springfield. p. 407.

BARKER, H. A. (1936 a), On the biochemistry of the methane fermentation. *Arch. Mikrobiol.* 7, 404.

— (1936 b), Studies upon the methane-producing bacteria. *Arch. Mikrobiol.* 7, 420.

— (1941), Studies on the methane fermentation. V. Biochemical activities of *Methanobacterium Omelianskii*. *J. biol. Chem.* 137, 153.

— (1944), On the rôle of carbon dioxide in the metabolism of *Clostridium thermoaceticum*. *Proc. nat. Acad. Sci. Wash.* 30, 88.

—, RUBEN, S. and BECK, J. V. (1940), *Proc. nat. Acad. Sci. Wash.* 26, 477. Cited in WERKMAN and WOOD (1942).

—, RUBEN, S. and KAMEN, M. (1940), *Proc. nat. Acad. Sci. Wash.* 26, 426. Cited in WERKMAN and WOOD (1942).

BARTH, L. G. (1929), The effects of acids and alkalies on the viscosity of protoplasm. *Protoplasma* 7, 505.

BAUER, E. und NAMEK, M. (1940), Über die Bildung von Kohlensäure-Estern und Carbonsäure-Estern. *Helv. chim. Acta* 23, 1101.

BECKER, Z. E. (1936), A comparison between the action of carbonic acid and other acids upon the living cell. *Protoplasma* 25, 161.

BENEDICT, S. R. (1931), The analysis of whole blood. II. The determination of sugar and of saccharoids (non-fermentable copper-reducing substances). *J. biol. Chem.* 92, 141.

BOHR, CHR. (1892), Beiträge zur Lehre von den Kohlensäureverbindungen des Blutes. *Skand. Arch. Physiol.* 3, 47.

— (1909), Blutgase und respiratorischer Gaswechsel. In NAGEL, W., Handbuch der Physiologie des Menschen. Braunschweig 1, 54.

BOOTH, N. (1930), The denaturation of proteins. VI. The titration of the basic and acidic groups in egg-albumin. Biochem. J. **24**, 158.

BOREI, H. (1942), Zur endogenen Atmung und Gärung der Oberhefe. I. Verhalten der Atmung gegen Fluorid and Azid. Biochem. Z. **312**, 160.

— (1943), Eine objektiv-spektralphotometrische Methode zur Phosphatbestimmung. Biochem. Z. **314**, 351.

— und LINDWALL, S. (1943), Zur endogenen Atmung und Gärung der Oberhefe. II. pH-Abhängigkeit der Atmung. Biochem. Z. **316**, 160.

— und SJÖDÉN, A. (1943), Züchtungsbedingte Veränderungen des Cytochrom c-Gehalts der Oberhefe. Ark. Kemi, Min. Geol. **16 A**, No. 19.

BRANDT, K. M. (1941 a), Über die Reservekohlenhydrate der Presshefe. Das Verhalten der Trehalose beim Wachstum und bei der Wärmeschädigung der Hefe. Biochem. Z. **309**, 190.

— (1941 b), Physiologische Chemie und Cytologie der Presshefe. (Ein Sammelbericht und neue Untersuchungen im ultravioletten Licht und an gefärbtem Material.) Protoplasma **36**, 77.

— (1942 a), Über Phosphatausscheidung und Permeabilität der getrockneten Bäckerhefe. Biochem. Z. **312**, 89.

— (1942 b), Der Phosphatgehalt der Presshefe bei aerobem Umsatz von Glucose und bei eintretendem Wachstum. Naturwiss. **30**, 278.

BRIGGS, A. P. (1924), Some applications of the colorimetric phosphate method. J. biol. Chem. **59**, 255.

BRINKMAN, R. (1933), The occurrence of carbonic anhydrase in lower marine animals. J. Physiol. **80**, 171.

— and MARGARIA, R. (1931), The influence of haemoglobin on the hydration and dehydration velocities of CO₂. J. Physiol. **72**, 6 P.

—, MARGARIA, R., MELDRUM, N. U. and ROUGHTON, F. J. W. (1932), The CO₂ catalyst present in blood. J. Physiol. **75**, 3 P.

BROOKS, J. and PACE, J. (1938), The distribution of carbon dioxide in the hen's egg. Proc. Roy. Soc. Lond. B. **126**, 196.

BROOKS, S. C. and BROOKS, M. M. (1941), The permeability of living cells. Protoplasma-Monographien. Berlin **19**.

BROWN, W. (1922), On the germination and growth of fungi at various temperatures and in various concentrations of oxygen and of carbon dioxide. Ann. Botany **36**, 257.

BUCHANAN, J. M., HASTINGS, A. B. and NESBETT, F. B. (1942), Glycogen formation from pyruvate *in vitro* in the presence of radioactive carbon dioxide. J. biol. Chem. **145**, 715.

BUCKMASTER, G. A. (1917), On the capacity of blood and haemoglobin to unite with carbon dioxide. J. Physiol. **51**, 164.

BUYTENDIJK, F. J. J. und WOERDEMAN, M. W. (1927), Die physico-chemischen Erscheinungen während der Entwicklung. I. Messung der Wasserstoffionenkonzentration. Arch. EntwMech. **112**, 387.

CARSON, S. F. and RUBEN, S. (1940), Proc. nat. Acad. Sci. Wash. **26**, 422. Cited in FOSTER, CARSON and RUBEN (1941).

CASPERSSON, T. (1941), Studien über den Eiweissumsatz der Zelle. *Naturwiss.* **29**, 33.

— und BRANDT, K. (1941), Nucleotidumsatz und Wachstum bei Presshefe. *Protoplasma* **35**, 507.

CHAMBERS, R. (1929), Hydrogen ion concentration of protoplasm. *Bull. nat. Res. Coun. Wash.* **69**, 37.

— and KERR, TH. (1932), Intracellular hydrion concentration studies. VIII. Cytoplasm and vacuole of *Limnobium* root-hair cells. *J. cell. comp. Physiol.* **2**, 105.

— and POLLACK, H. (1927), Mierurgical studies in cell physiology. IV. Colorimetric determination of the nuclear and cytoplasmic pH in the starfish egg. *J. gen. Physiol.* **10**, 739.

CHANDLER, R. C. (1941), Nature of bound water in colloidal systems. *Plant Physiol.* **16**, 273.

CHAPIN, C. W. (1918), Carbon dioxide in the primary cultivation of the gonococcus. *J. infect. Dis.* **23**, 342.

CHASE, A. M. and GLASER, O. (1930), Forward movement of Paramaccaium as a function of the hydrogen ion eoneentration. *J. gen. Physiol.* **13**, 627.

CLAASEN, H. (1934), Ueber die Vorgänge bei dem Aufbau der Hefekörpermasse und die theoretisch und praktisch möglichen Höchsternten an Hefe aus Zuckerlösungen. *Z. Ver. dtsch. Zuckerind., Techn.* **T. 84**, 713.

CLOWES, G. H. A. and SMITH, H. W. (1922), Carbon dioxide as an inhibitor of cell growth. *J. biol. Chem.* **50**, IV.

COHEN, M. B. and FLEMING, J. S. (1918), The diagnosis of epidemic meningitis and the control of its treatment by rapid bacteriologic and serologic methods. *J. infect. Dis.* **23**, 337.

CONWAY, E. J. and BYRNE, A. (1933), An absorption apparatus for the micro-determination of certain volatile substances. I. The micro-determination of ammonia. *Biochem. J.* **27**, 419.

— and FEARON, P. J. (1944), Acid-labile carbon dioxide in mammalian muscle and the hydrogen ion concentration of the muscle fibre. *Nature* **153**, 54.

—, O'BRIEN, M. F. and BOYLE, P. J. (1941), Interchange of the ammonium and potassium ions in muscle and yeast. *Nature* **148**, 662.

CRAIG, F. N. and BEECHER, H. K. (1943), The effect of carbon dioxide tension on tissue metabolism. *J. gen. Physiol.* **26**, 473. Cited in *Chem. Abstr.* **37**, 4452 (1943).

CULLEN, G. E., KEELER, H. R. and ROBINSON, H. W. (1925), The pK' of the Henderson-Hasselbalch equation for hydrion concentration of serum. *J. biol. Chem.* **66**, 301.

DANIELLI, J. F. (1941), On the pH at the surface of ovalbumin molecules, and the protein error with indicators. *Biochem. J.* **35**, 470.

DANIELSON, I. S., CHU, H. I. and HASTINGS, A. B. (1939), The pK_1' of carbonic acid in concentrated protein solutions and muscle. *J. biol. Chem.* **131**, 243.

— and HASTINGS, A. B. (1939), A method for determining tissue carbon dioxide. *J. biol. Chem.* **130**, 349.

DAVIES, R. (1940), The effect of carbon dioxide on the growth of the tubercle bacillus. *Brit. J. exp. Path.* 21, 243.

DENOLIN, H. (1938), Travaux récents sur le transport de l'anhydride carbonique par le sang. *Ann. Soc. Sci. méd. nat. Brux.* 3—4, 23.

DICKENS, F. (1940), Die manometrische Methode. In BAMANN, E. und MYRBÄCK, K., *Die Methoden der Fermentforschung*. Leipzig. p. 985.

DILL, D. B., DALY, C. and FORBES, W. H. (1937), The p_k' of serum and red cells. *J. biol. Chem.* 117, 569.

DIXON, M. (1943), *Manometric methods as applied to the measurement of cell respiration and other processes*. Second Edition. Cambridge.

DORFMAN, V. A. (1938), The pH and anaerobic redox potential in early amphibian morphogenesis. *Bull. Biol. Méd. exp. URSS.* 6, 413.

DUBOISSEON, M. (1939), Studies on the chemical processes which occur in muscle before, during and after contraction. *J. Physiol.* 94, 461.

ELSDEN, S. R. (1938), The effect of CO_2 on the production of succinic acid by *Bact. coli commune*. *Biochem. J.* 32, 187.

ENGELMANN, TH. W. (1883), *Bacterium photometricum*. *Pflüg. Arch. ges. Physiol.* 30, 95.

EVANS, E. A. JR. (1942), Carbon dioxide utilization in animal tissues. *Science* 96, 25.

— and SLOTIN, L. (1940 a), The utilization of carbon dioxide in the synthesis of α -ketoglutaric acid. *J. biol. Chem.* 136, 301.

— and SLOTIN, L. (1940 b), The rôle of carbon dioxide in the synthesis of urea in rat liver slices. *J. biol. Chem.* 136, 805.

— and SLOTIN, L. (1941), *J. biol. Chem.* 141, 439. Cited in WERKMAN and WOOD (1942).

—, SLOTIN, L. and VENNESLAND, B. (1942), Carbon dioxide assimilation in cell-free liver extracts. *J. biol. Chem.* 143, 565.

—, VENNESLAND, B. and SLOTIN, L. (1943), Mechanism of carbon dioxide fixation in cell-free extracts of pigeon liver. *J. biol. Chem.* 147, 771.

FAURHOLT, C. (1924), Études sur les solutions aqueuses d'anhydride carbonique et d'acide carbonique. *J. Chim. phys.* 21, 400.

— (1925), Études sur les solutions aqueuses de carbamates et de carbonates. *J. Chim. phys.* 22, 1.

FENN, W. O. and MAURER, F. W. (1935), The pH of muscle. *Protопlasma* 24, 337.

FERGUSON, J. K. W., HORVATH, S. M. and PAPPENHEIMER, J. R. (1938), The transport of carbon dioxide by erythrocytes and plasma in dogfish blood. *Biol. Bull. Wood's Hole* 75, 381.

— and ROUGHTON, F. J. W. (1934 a), The direct chemical estimation of carbamino compounds of CO_2 with haemoglobin. *J. Physiol.* 83, 68.

— and ROUGHTON, F. J. W. (1934 b), The chemical relationships and physiological importance of carbamino compounds of CO_2 with haemoglobin. *J. Physiol.* 83, 87.

FICHTER, F.R. und BECKER, B. (1911), Über die Bildung symmetrisch dialkylierter Harnstoffe durch Erhitzen der entsprechenden Carbamate. Ber. dtsch. chem. Ges. 44, 3481.

FIFE, J. M. and FERGUSON, W. C. (1941), The effect of high concentrations of carbon dioxide on the glutamine and asparagine metabolism of the sugar beet. Plant Physiol. 16, 677.

— and FRAMPTON, V. L. (1935), The effect of carbon dioxide upon the pH and certain nitrogen fractions of the sugar-beet plant. J. biol. Chem. 109, 643.

FISCHER, R. A. (1944), Statistical methods for research workers. Edinburgh.

FOSTER, J. W. (1940), The rôle of organic substrates in photosynthesis of purple bacteria. J. gen. Physiol. 24, 123.

—, CARSON, S. F. and RUBEN, S. (1941), Spiritus Vitalis. Chron. bot. 6, 337.

—, CARSON, S. F., RUBEN, S. and KAMEN, M. D. (1941), Radioactive C as an indicator of CO₂ utilization. VII. The assimilation of CO₂ by molds. Proc. nat. Acad. Sci. Wash. 27, 590.

FRASER, L. M. (1925), A comparison of the effects on the isolated beating intestine of CO₂ and of a mineral acid. Amer. J. Physiol. 72, 119.

FREEMAN, M. E. (1942), Heat capacity and bound water in starch suspensions. Arch. Biochem. New York 1, 27. Cited in Chem. Abstr. 37, 297 (1943).

FREUDENBERG, K. und PLANKENHORN E. (1938), Synthese der 2, 4, 6-Trimethylglucose und ihre Beziehung zum Hefeglucan. Liebigs Ann. 536, 257.

FRIEDEMANN, E. T. and KLAAS, R. (1936), The determination of ethyl alcohol. J. biol. Chem. 115, 47.

FROMAGEOT, CL. und DESNUELLE, P. (1935), Eine neue Methode zur Bestimmung der Brenztraubensäure. Biochem. Z. 279, 174.

GEFFCKEN, G. (1904), Beiträge zur Kenntnis der Löslichkeitsbeeinflussung. Z. phys. Chemie 49, 257.

GENAUD, M. P. (1929), Les échanges d'ions entre cellules de levures et solutions de chlorure d'ammonium. C. R. Acad. Sci. Paris 188, 1513.

GLADSTONE, G. P., FILDES, P. and RICHARDSON, G. M. (1935), Carbon dioxide as an essential factor in the growth of bacteria. Brit. J. exp. Path. 16, 335.

GOEBEL, W. F. (1927), On the oxidation of glucose in alkaline solutions of iodine. J. biol. Chem. 72, 801.

GOLDING, N. S. (1940), The gas requirements of molds. III. The effect of various concentrations of carbon dioxide on the growth of *Penicillium roqueforti* in air. J. dairy. Sci. 23, 891.

GUYE, PH.-A. et PINTZA, A. (1908), Détermination des densités des gaz anhydride carbonique, ammoniac et protoxyde d'azote. Mém. Soc. Phys. Hist. nat. Genève 35, 551.

HAMON, F. (1936), Influence de l'acide carbonique sur la respiration des tissus végétaux et de la levure. Ann. Physiol. Physicochimie Biol. 12, 940.

HASSID, W. Z., JOSLYN, M. A. and McCREADY, R. M. (1941), The molecular constitution of an insoluble polysaccharide from yeast, *Saccharomyces cerevisiae*. *J. Amer. chem. Soc.* **63**, 295.

HASTINGS, A. B. and KISTIAKOWSKY, G. B. (1940), *Conf. Appl. Nucl. Phys.* Cambridge. Cited in FOSTER, CARSON and RUBEN (1941).

—, SENDROY, J. Jr. and VAN SLYKE, D. D. (1928), Studies of gas and electrolyte equilibria in blood. The value of pK' in the Henderson-Hasselbalch equation for blood serum. *J. biol. Chem.* **79**, 183.

HAWORTH, W. N., HEATH, R. L. and PEAT, S. (1941), The constitution of yeast mannan. *J. chem. Soc. Lond.* I, 833.

—, HIRST, E. L. and ISHERWOOD, F. A. (1937), Polysaccharides, XXIV. Yeast mannan. *J. chem. Soc. Lond.* I, 784.

HAYWOOD, Ch. (1925), The relative importance of pH and carbon dioxide tension in determining the cessation of ciliary movement in acidified sea water. *J. gen. Physiol.* **7**, 693.

— (1927), Carbon dioxide as a narcotic agent. *Biol. Bull. Wood's Hole* **53**, 450.

— and Root, W. S. (1930), A quantitative study of the effect of carbon dioxide upon the cleavage rate of the Arbacia egg. *Biol. Bull. Wood's Hole* **59**, 63.

HEILMANN, F. (1929), Einfluss der Kohlensäure auf Heterotrophen. *Diss. Göttingen.*

HENRIQUES, O. M. (1928 a), Die Bindungsweise des Kohlendioxyds im Blute. I. Über die Geschwindigkeiten der Anhydrierung bzw. der Hydratisierung der Kohlensäurekomponenten im Blute. *Biochem. Z.* **200**, 1.

— (1928 b), Die Bindungsweise des Kohlendioxyds im Blute. II. Der experimentelle Nachweis schnell reagierenden, gebundenen CO_2 im Hämoglobin. *Biochem. Z.* **200**, 5.

— (1928 c), Die Bindungsweise des Kohlendioxyds im Blute. III. Der experimentelle Nachweis eines CO_2 -Hämoglobinkomplexes in Lösungen von CO_2 und Hämoglobin. *Biochem. Z.* **200**, 10.

— (1928 d), Die Bindungsweise des Kohlendioxyds im Blute. IV. Das CO_2 -Bindungsvermögen von reduziertem und Oxyhämoglobin. *Biochem. Z.* **200**, 18.

— (1928 e), Die Bindungsweise des Kohlendioxyds im Blute. V. Einige physiologische Betrachtungen über das Carbhämoglobinproblem. *Biochem. Z.* **200**, 22.

— (1929), Über Carbhämoglobin. *Ergebn. Physiol.* **28**, 625.

— (1931), Über die Zustandsformen des Kohlendioxyds im Blut. *Biochem. Z.* **243**, 241.

HERAEUS, W. (1886), Ueber das Verhalten der Bacterien im Brunnenwasser sowie über reducirende und oxydirende Eigenschaften der Bacterien. *Z. Hyg. InfektKr.* **1**, 193.

HES, J. W. (1938), Function of carbon dioxide in the metabolism of heterotrophic cells. *Nature* **141**, 647.

HILL, D. K. (1940), Hydrogen-ion concentration changes in frog's muscle following activity. *J. Physiol.* **98**, 467.

JACOBS, M. H. (1920 a), To what extent are the physiological effects of carbon dioxide due to hydrogen ions? Amer. J. Physiol. 51, 321.

— (1920 b), The production of intracellular acidity by neutral and alkaline solutions containing carbon dioxide. Amer. J. Physiol. 53, 457.

— (1922), The effects of carbon dioxide on the consistency of protoplasm. Biol. Bull. Wood's Hole 42, 14.

JOHNSON, M. J. (1941), The rôle of aerobic phosphorylation in the Pasteur effect. Science 94, 200.

JUKES, T. H. (1934), The electrometric titration of lecithin and cephalin. J. biol. Chem. 107, 783.

JUST, F. (1940), Über die rechnerische und experimentelle Ermittlung der zwischen den Zellen in abgepresster Hefe zurückbleibenden Gär- und Züchtungsflüssigkeitsmengen. Biochem. Z. 306, 33.

KALCKAR, H. M. (1942), The function of phosphate in cellular assimilations. Biol. Rev. 17, 28.

KALNITSKY, G. and WERKMAN, C. H. (1942), Fixation of CO₂ by a cell-free extract of Escherichia coli. J. Bact. 44, 256.

KEILIN, D. and HARTREE, E. F. (1939), Cytochrome and cytochrome oxidase. Proc. Roy. Soc. Lond. B. 127, 167.

— and MANN, T. (1940), Carbonic anhydrase. Purification and nature of the enzyme. Biochem. J. 34, 1163.

KLEINZELLER, A. (1941), The formation of succinic acid in yeast. Biochem. J. 35, 495.

KLUYVER, A. J. (1939), Die Kohlensäure im Stoffwechsel der Lebewesen. Suom. Kemistilehti 12 A, 81.

— und DONKER, H. J. L. (1926), Die Einheit in der Biochemie. Chem. Zelle, Gewebe 13, 134.

— und VAN ROOSMALEN, F. L. W. (1932), Über die Vergärung der Trehalose. Biochem. Z. 245, 13.

KNIGHT, B. C. J. G. (1936), Bacterial nutrition. Med. Res. Comm., Spec. Rep. Ser. Lond. 210.

KOCH, M. and KOCH, F. C. (1925), The presence of trehalose in yeast. Science 61, 570.

KÖGL, F. und BORG, W. A. J. (1941), Hefewachstum, Gärung und Faktor Z-Wirkung. Hoppe-Seyl. Z. 269, 97.

KÖHLER, A. (1904), Mikrophotographische Untersuchungen mit ultraviolettem Licht. Z. wiss. Mikr. 21, 129 und 273.

— und TOGBY, A. F. (1928), Mikroskopische Untersuchungen einiger Augenmedien mit ultraviolettem und mit polarisiertem Licht. Arch. Augenheilk. 99, 263.

KOLTHOFF, I. M. und MENZEL, H. (1931), Die Massanalyse. 2. Aufl., II. Teil. Berlin.

KRAMPITZ, L. O. and WERKMAN, C. H. (1941), The enzymic decarboxylation of oxaloacetate. Biochem. J. 35, 595.

—, WOOD, H. G. and WERKMAN, C. H. (1943), J. biol. Chem. 147, 243. Cited in EVANS, E. A. Jr., Ann. Rev. Biochem. 13, 187 (1944).

KREBS, H. A. (1943), Carbon dioxide assimilation in heterotrophic organisms. Ann. Rev. Biochem. 12, 529.

— and EGGLESTON, L. V. (1940), Biological synthesis of oxaloacetic acid from pyruvic acid and carbon dioxide. Biochem. J. 34, 1383.

— and EGGLESTON, L. V. (1941), Biological synthesis of oxaloacetic acid from pyruvic acid and carbon dioxide. II. The mechanism of carbon dioxide fixation in propionic acid bacteria. Biochem. J. 35, 676.

— und HENSELEIT, K. (1932), Untersuchungen über die Harnstoffbildung im Tierkörper. Hoppe-Seyl. Z. 210, 33.

KROGH, A. (1919), The rate of diffusion of gases through animal tissues, with some remarks on the coefficient of invasion. J. Physiol. 52, 391.

LAGONI, H. (1939), Über die Wirkung von Sauerstoff, Kohlendioxyd und Wasserstoff auf das Wachstum und den Stoffwechsel von *Bact. coli*. Vorratspfl. LebMittelforsch. 2, 167. Cited in Ber. ges. Physiol. 114, 334 (1939).

LANDOLT-BÖRNSTEIN (1923—1936), Physikalisch-chemische Tabellen, V. Aufl. Berlin.

LARSON, W. P., HARTZELL, T. B. and DIEHL, H. S. (1918), The effect of high pressures on bacteria. J. infect. Dis. 22, 271.

LASER, H. and ROTHSCHILD, L. (1940), The metabolism of the eggs of *Psammechinus miliaris* during the fertilization reaction. Proc. Roy. Soc. Lond. B. 126, 539.

LEUTHARDT, F. (1940), Über die Bestimmung der Reaktion, der Titrationskurve und der Kohlensäurebindungskurve von Geweben. Biochem. Z. 306, 399.

LIESKE, R. und HOFMANN, E. (1929), Untersuchungen über Hefegärung bei hohen Gasdrucken. Biochem. Z. 210, 448.

LINDAHL, P. E. (1940), Über die CN-resistente Atmung des Seeigeleies. Ark. Kemi, Min. Geol. 14 A, No. 12.

LIPMANN, F. (1941), Metabolic generation and utilization of phosphate bond energy. Advances Enzymol. 1, 99.

— und MEYERHOF, O. (1930), Über die Reaktionsänderung des tätigen Muskels. Biochem. Z. 227, 84.

LISON, L. (1941), ph et rh intracellulaires. Tabulae biologicae 19 (Cellula) Haag II, 1.

LOPRIORE, G. (1895), Ueber die Einwirkung der Kohlensäure auf das Protoplasma der lebenden Pflanzenzelle. Jahrb. wiss. Bot. 28, 531.

LORBER, V., HEMINGWAY, A. and NIER, A. O. (1943), Assimilation of carbon dioxide by the isolated mammalian heart. J. biol. Chem. 151, 647.

LÜERS, H. (1929), Chemie des Brauwesens. Berlin.

LYNEN, F. (1941), Über den aeroben Phosphatbedarf der Hefe. Ein Beitrag zur Kenntnis der Pasteur'schen Reaktion. Liebigs Ann. 546, 120.

MAHDIHASSAN, S. (1930), Die Wasserstoffionenkonzentration im Zellinnern von *Fusarium lini* B. und Hefe. Biochem. Z. 226, 203.

MAISON, G. L., ORTH, O. S. and LEMMER, K. E. (1938), pH changes in rabbit and human striated muscle after contraction. Amer. J. Physiol. 121, 311.

MARGARIA, R. (1931 a), The effect of CO₂ on vapour pressure of blood and of haemoglobin solutions. J. Physiol. 72, 7 P.

— (1931 b), On the state of CO₂ in blood and haemoglobin solutions, with an appendix on some osmotic properties of glycine in solution. J. Physiol. 73, 311.

— and GREEN, A. A. (1933), The first dissociation constant, pK_1' , of carbonic acid in hemoglobin solutions and its relation to the existence of a combination of hemoglobin with carbon dioxide. J. biol. Chem. 102, 611.

— und v. MURALT, A. (1934), Photoelektrische Messung der pH-Änderung im Muskel während der Kontraktion. Naturwiss. 22, 634.

MARKHAM, A. E. and KOBE, K. A. (1941), The solubility of carbon dioxide and nitrous oxide in aqueous salt solutions. J. Amer. chem. Soc. 63, 449.

MASUR, B. L. (1926), Zur Biologie des Tuberkelbazillus. Zbl. Bakt. I. Abt. Orig. 99, 46.

MAYER, P. (1923), Zur Methodik der quantitativen Bestimmung des Hefeglykogens und zur Frage der Alkaliwirkung auf die Glykogenbildung in der Hefe. Biochem. Z. 136, 487.

McALPINE, J. G. and SLANETZ, CH. A. (1928), Studies on the metabolism of the Abortus-Melitensis group. 4. Effect of various concentrations of carbon dioxide. J. infect. Dis. 43, 232.

MCANALLY, R. A. and SMEDLEY-MACLEAN, I. (1935), The synthesis of reserve carbohydrate by yeast. I. Synthesis from glucose and maltose and the influence of phosphate thereon. Biochem. J. 29, 1872.

MELDRUM, N. U. and ROUGHTON, F. J. W. (1932), Some properties of carbonic anhydrase, the CO₂ enzyme present in blood. J. Physiol. 75, 15 P.

— and ROUGHTON, F. J. W. (1933 a), Carbonic anhydrase. Its preparation and properties. J. Physiol. 80, 113.

— and ROUGHTON, F. J. W. (1933 b), The state of carbon dioxide in blood. J. Physiol. 80, 143.

MELLANBY, J. and THOMAS, C. J. (1920), The carbon dioxide carrying power of the constituents of plasma. The alkali reserve of blood. J. Physiol. 54, 178.

MEYER, A. (1904), Orientierende Untersuchungen über Verbreitung, Morphologie und Chemie des Volutins. Bot. Ztg. 62, 113.

MEYERHOF, O. und IWASAKI, K. (1930), Über Beeinflussung der Gärungsgröße und des Oxydationsquotienten der Hefe. Biochem. Z. 226, 16.

— und LOHMANN, K. (1926), Über die Vorgänge bei der Muskelermüdung. Biochem. Z. 168, 128.

MIRSKI, A. und WERTHEIMER, E. (1939), Zuckerassimilation durch lebende Hefezellen. Über die in den ersten Minuten nachweisbaren Veränderungen. Enzymologia 7, 58.

MOLISCH, H. (1907), Die Purpurbakterien nach neuen Untersuchungen. Jena.

MUSFELD, W. (1942), Versuche über die Aufnahme von Zucker durch Hefzellen. Ber. Schweiz. bot. Ges. 52, 583.

MYRBÄCK, K. (1936), Trehalos i pressjäst. Svensk kem. Tidskr. 48, 55.

— und VALLIN, I. (1944), Vitamin B₁ in der Presshefe: Über Atmung und Gärung der Hefe. Svensk kem. Tidskr. 56, 400.

— und ÖRTENBLAD, B. (1936), Trehalose und Hefe. I. Biochem.Z. 288, 329.

— und ÖRTENBLAD, B. (1937 a), Trchalose und Hefe. II. Biochem.Z. 291, 61.

— und ÖRTENBLAD, B. (1937 b), Trehalose und Hefe. III. Biochem.Z. 292, 230.

NEGELEIN, E. und BRÖMEL, H. (1939), R-Diphosphoglycerinsäure, ihre Isolierung und Eigenschaften. Biochem.Z. 303, 132.

NILSSON, R. und ELANDER, M. (1941), Zur Kenntnis der alkoholischen Gärung in dem intakten Fermentsystem der Hefezelle und in desorganisierten Zymasesystemen. V. Biochem.Z. 309, 51.

NOWAK, J. (1908), Le bacille de Bang et sa biologie. Ann. Inst. Pasteur 22, 541.

PARNAS, J. K. und HELLER, J. (1924), Über den Ammoniakgehalt und über die Ammoniakbildung im Blute. Biochem.Z. 152, 1.

PETERS, J. P. and VAN SLYKE, D. D. (1932), Quantitative clinical chemistry. London.

PETT, L. B. (1936), Studies on yeast grown in cyanide. II. Biochem. J. 30, 1438.

PLATZ, G. A., DURRELL, L. W. and HOWE, M. F. (1927), Effect of carbon dioxide upon the germination of Chlamydospores of Ustilago Zeae (Beckm.). J. agric. Res. 34, 137.

PULVER, R. und VERZÁR, F. (1940), Der Zusammenhang von Kalium- und Kohlehydratstoffwechsel bei der Hefe. Helvet. chim. Acta 23, 1087.

QUASTEL, J. H. and WHEATLEY, A. H. M. (1932), The relation of thiol compounds to glucose fermentation. Biochem. J. 26, 2169.

RAHN, O. (1941), Protozoa need carbon dioxide for growth. Growth 5, 197. Cited in Biol. Abstr. 22856 (1941).

REISS, P. (1926), Le pH intérieur cellulaire. Paris.

RIES, E. (1938), Grundriss der Histophysiologie. Leipzig.

RIPPEL, A. und BORTELS, H. (1927), Vorläufige Versuche über die allgemeine Bedeutung der Kohlensäure für die Pflanzenzelle. (Versuch an Aspergillus niger). Biochem. Z. 184, 237.

— und HEILMANN, F. (1930), Quantitative Untersuchungen über die Wirkung der Kohlensäure auf Heterotrophen. Arch. Mikrobiol. 1, 119.

RITTENBERG, D. and WAELSCH, H. (1940), The source of carbon for urea formation. J. biol. Chem. 136, 799.

ROBISON, R. und MACFARLANE, M. G. (1940), Biologisch wichtige Derivate der Zucker. Zwischen- und Endprodukte beim Abbau.

In BAMANN, E. und MYRBÄCK, K., Die Methoden der Fermentforschung. Leipzig. p. 296.

— and MORGAN, W. T. J. (1928), Trehalosemonophosphoric ester isolated from the products of fermentation of sugars with dried yeast. Biochem. J. 22, 1277.

ROCKWELL, G. E. and HIGHBERGER, J. H. (1927), The necessity of carbon dioxide for the growth of bacteria, yeasts and molds. J. infect. Dis. 40, 438.

ROELOFSEN, P. A. (1935), On photosynthesis of the Thiorhodaceae. Diss. Utrecht.

ROOT, W. S. (1930), The influence of carbon dioxide upon the oxygen consumption of *Paramaecium* and the egg of *Arbacia*. Biol. Bull. Wood's Hole 59, 48.

ROUGHTON, F. J. W. (1935), Recent work on carbon dioxide transport by the blood. Physiol. Rev. 15, 241.

RUBEN, S. (1943), Photosynthesis and phosphorylation. J. Amer. chem. Soc. 65, 279.

— and KAMEN, M. D. (1940 a), Photosynthesis with radioactive carbon. IV. Molecular weight of the intermediate products and a tentative theory of photosynthesis. J. Amer. chem. Soc. 62, 3451.

— and KAMEN, M. D. (1940 b), Proc. nat. Acad. Sci. Wash. 26, 418. Cited in FOSTER, CARSON and RUBEN (1941).

—, KAMEN, M. D., HASSID, W. Z. and DE VAULT, D. C. (1939), Photosynthesis with radio-carbon. Science 90, 570.

—, RANDALL, M., KAMEN, M. and HYDE, J. L. (1941), Heavy oxygen (O^{18}) as a tracer in the study of photosynthesis. J. Amer. chem. Soc. 63, 877.

RUNNSTRÖM, J., BOREI, H. und SPERBER, E. (1939), Wo setzt die Hemmung der Hefeatmung durch Fluorid ein? Ark. Kemi, Min. Geol. 13 A, No. 22.

— und BRANDT, K. (1941), Über den Einfluss von Kohlenmonoxyd, Cystein, Glutathion, Jodessigsäure und Fluorid auf den Umsatz der Brenztraubensäure durch Bäckerhefe. Ark. Kemi, Min. Geol. 15 A, No. 6.

—, BRANDT, K. und MARCUSE, R. (1940), Die Assimilation von Ammoniak durch Bäckerhefe unter aeroben und anaeroben Bedingungen. Ark. Kemi, Min. Geol. 14 B, No. 8.

—, BRANDT, K. und MARCUSE, R. (1943), Über den Umsatz der Brenztraubensäure durch destruierte Bäckerhefe. Ark. Kemi, Min. Geol. 17 A, No. 3.

— und SPERBER, E. (1938 a), Zur Kenntnis der Beziehungen zwischen Permeabilität und Stoffwechsel der Hefezelle. Biochem. Z. 298, 340.

— und SPERBER, E. (1938 b), Action of cystein on respiration, fermentation and synthesis in yeast cells. Nature 141, 689.

— und SPERBER, E. (1941), Zur Kenntnis des Umsatzes der Brenztraubensäure durch Bäckerhefe. Ark. Kemi, Min. Geol. 15 A, No. 5.

RUNNSTRÖM, J., SPERBER, E. und KARLSSON, E. (1939), Über die Permeabilität der Zellen der Bäckerhefe für Brenztraubensäure unter verschiedenen physiologischen Bedingungen. *Ark. Kemi, Min. Geol.* 13 B, No. 10.

SCHABADUSCH, A. L. (1937), Morphology of glycogen distribution and transformation. *Bull. Biol. Méd. exp. URSS.* 4, 13.

SCOTT, D. A. and MENDIVE, J. R. (1941), Chemical observations on carbonic anhydrase. *J. biol. Chem.* 140, 445.

SEARLE, D. S. and REINER, L. (1940), Effect of carbon dioxide on glucose metabolism of trypanosomes. *Proc. Soc. exp. Biol. Med.* 43, 80.

— and REINER, L. (1941), *J. biol. Chem.* 141, 563. Cited in WERKMAN and Wood (1942).

SEVAG, M. G., CATTANEO, C. und MAIWEG, L. (1935), Über die Natur der Hefe-Polysaccharide. *Liebigs Ann.* 519, 111.

SHAUGHNESSY, H. J. (1939), A method for producing increased carbon dioxide tension in individual culture tubes and flasks. *J. Bact.* 37, 153.

SIDWELL, A. E. Jr., MUNCH, R. H., BARRON, E. S. G. and HOGNESS, T. R. (1938), The salt effect on the hemoglobin-oxygen equilibrium. *J. biol. Chem.* 123, 335.

SIEGFRIED, M. (1905), Über die Bindung von Kohlensäure durch amphotere Amidokörper. *Hoppe-Seyl. Z.* 44, 85.

— (1906), Über die Bindung von Kohlensäure durch amphotere Amidokörper. II. *Hoppe-Seyl. Z.* 46, 401.

— und HOWWJANZ, S. (1909), Über die Bindung von Kohlensäure durch Alkohole, Zucker und Oxsäuren. *Hoppe-Seyl. Z.* 59, 376.

— und LIEBERMANN, H. (1908), Über die Bindung von Kohlensäure durch amphotere Aminokörper. IV. *Hoppe-Seyl. Z.* 54, 437.

— und NEUMANN, C. (1908), Über die Bindung von Kohlensäure durch amphotere Aminokörper. III. *Hoppe-Seyl. Z.* 54, 423.

SLADE, H. D., WOOD, H. G., NIER, A. O., HEMINGWAY, A. and WERKMAN, C. H. (1942), Assimilation of heavy carbon dioxide by heterotrophic bacteria. *J. biol. Chem.* 143, 133.

SMALL, J. (1929), Hydrogen-ion concentration in plant cells and tissues. *Protoplasma-Monographien.* Berlin 2.

SMITH, H. W. (1926), The action of acids on turtle heart muscle with reference to the penetration of anions. *Amer. J. Physiol.* 76, 411.

— and CLOWES, G. H. A. (1924), The influence of carbon dioxide on the velocity of division of marine eggs. *Amer. J. Physiol.* 68, 183.

SMITH, J. H. C. and COWIE, D. B. (1941), Absorption and utilization of radioactive carbon dioxide by sunflower leaves. *Plant. Physiol.* 16, 257.

SMITH, T. (1924), Some cultural characters of *Bacillus Abortus* (Bang) with special reference to CO_2 requirements. *J. exp. Med.* 40, 219.

SMYTH, D. H. (1940), Vitamin B_1 and the synthesis of oxaloacetate by *Staphylococcus*. *Biochem. J.* 34, 1598.

SMYTHE, C. V. (1939), The effect of certain tissue extracts, of ammonia salts and of certain amides on the rate of fermentation by baker's yeast. *Enzymologia* 6, 9.

SÖHNGEN, N. L. (1906), Het ontstaan en verdwijnen van waterstof en methaan onder den invloed van het organische leven. Diss. Delft.

SOLOMON, A. K., VENNESLAND, B., KLEMPERER, F. W., BUCHANAN, J. M. and HASTINGS, A. B. (1941), The participation of carbon dioxide in the carbohydrate cycle. *J. biol. Chem.* **140**, 171.

SPEK, J. (1937), Das pH in der lebenden Zelle. *Ergebn. Enzymforsch.* **6**, 1.

— und CHAMBERS, R. (1933), Das Problem der Reaktion des Protoplasmas. (Neue experimentelle Studien, ausgeführt an Amoeben). *Protoplasma* **20**, 376.

SPERBER, E. und RUNNSTRÖM, J. (1939), Über die Konkurrenz einiger Substrate nm die Enzyme der lebenden Zellen der Bäckerhefe. *Biochem. Z.* **300**, 373.

STADIE, W. C. and HAWES, E. R. (1928), Studies on the oxygen-, acid-, and base-combining properties of blood. IV. The apparent first dissociation constant, pK_1' , of carbonic acid and the activity coefficient of the bicarbonate ion in solutions of hemoglobin, methemoglobin, cyanhemoglobin, and nitric oxide hemoglobin at varying ionic strengths. *J. biol. Chem.* **77**, 265.

— and O'BRIEN, H. (1933), The catalysis of the hydration of carbon dioxide and dehydration of carbonic acid by an enzyme isolated from red blood cells. *J. biol. Chem.* **103**, 521.

— and O'BRIEN, H. (1936), The carbamate equilibrium. I. The equilibrium of amino acids, carbon dioxide, and carbamates in aqueous solution; with a note on the Ferguson-Roughton carbamate method. *J. biol. Chem.* **112**, 723.

— and O'BRIEN, H. (1937), The carbamate equilibrium. II. The equilibrium of oxyhemoglobin and reduced hemoglobin. *J. biol. Chem.* **117**, 439.

STEINER, A. and CORI, C. F. (1935), The preparation and determination of trehalose in yeast. *Science* **82**, 422.

STEPHENSON, M. and STICKLAND, L. H. (1932), Hydrogenlyases. Bacterial enzymes liberating molecular hydrogen. *Biochem. J.* **26**, 712.

— and STICKLAND, L. H. (1933), Hydrogenase. III. The bacterial formation of methane by the reduction of one-carbon compounds by molecular hydrogen. *Biochem. J.* **27**, 1517.

STIER, T. J. B. and NEWTON, M. I. (1939), Changes in the rate of respiration of baker's yeast during assimilation. *J. cell. comp. Physiol.* **13**, 343.

— and SPRINCE, H. (1941), Carbohydrate anabolism and catabolism of "starved" baker's yeast cells. *J. cell. comp. Physiol.* **18**, 135.

— and STANNARD, J. N. (1936), A kinetic analysis of the endogenous respiration of baker's yeast. *J. gen. Physiol.* **19**, 461.

TAIT, A. and FLETCHER, L. (1926), The development and nutrition of yeast. III. The lag phase and associated phenomena. *J. Inst. Brewing* **32**, 385.

TAMIYA, H. und KUBO, H. (1938), Zur Frage der Nichtidentität der Cytochromoxydase mit dem sauerstoffübertragenden Ferment von O. Warburg. *Acta phytochim. Tokyo* **10**, 317.

TANRET, G. (1931), Sur le tréhalose de la levure. C. R. Acad. Sci. Paris. 192, 1056.

TEORELL, T. (1932), Eine neue Methode zur titrimetrischen Bestimmung kleinster Mengen Ammoniak. Biochem. Z. 248, 246.

THIEL, A. (1913 a), Über die langsame Neutralisation der Kohlensäure. Ber. dtsch. chem. Ges. 46, 241.

— (1913 b), Das Wesen der Zeitreaktion zwischen Kohlensäure und Basen. Ber. dtsch. chem. Ges. 46, 867.

THORNTON, N. C. (1933 a), Carbon dioxide storage. III. The influence of carbon dioxide on the oxygen uptake by fruits and vegetables. Contr. Boyce Thompson Inst. 5, 371.

— (1933 b), Carbon dioxide storage. IV. The influence of carbon dioxide on the acidity of plant tissue. Contr. Boyce Thompson Inst. 5, 403.

VALLEY, G. (1928), The effect of carbon dioxide on bacteria. Quart. Rev. Biol. 3, 209.

— and RETTGER, L. F. (1926), Carbon dioxide requirements of bacteria. J. Bact. 11, 78.

— and RETTGER, L. F. (1927), The influence of carbon dioxide on bacteria. J. Bact. 14, 101.

VAN GOOR, H. (1940), Die Verbreitung und Bedeutung der Karbon-anhydrase. Enzymologia 8, 113.

VAN NIEL, C. B. (1931), On the morphology and physiology of the purple and green sulphur bacteria. Arch. Mikrobiol. 3, 1.

— (1941), The bacterial photosynthesis and their importance for the general problem of photosynthesis. Advances Enzymol. 1, 263.

—, THOMAS, J. O., RUBEN, S. and KAMEN, M. D. (1942), Radioactive carbon as an indicator of carbon dioxide utilization. IX. The assimilation of carbon dioxide by protozoa. Proc. nat. Acad. Sci. Wash. 28, 157. Cited in Chem. Abstr. 36, 5264 (1942).

VAN SLYKE, D. D., HASTINGS, A. B., MURRAY, C. D. and SENDROY, J. Jr. (1925), Studies of gas and electrolyte equilibria in blood. VIII. The distribution of hydrogen, chloride, and bicarbonate ions in oxygenated and reduced blood. J. biol. Chem. 65, 701.

— and HAWKINS, J. A. (1930), Studies of gas and electrolyte equilibria in blood. XVI. The evolution of carbon dioxide from blood and buffer solutions. J. biol. Chem. 87, 265.

—, SENDROY, J. Jr., HASTINGS, A. B. and NEILL, J. M. (1928), Studies of gas and electrolyte equilibria in blood. X. The solubility of carbon dioxide at 38° in water, salt solution, serum, and blood cells. J. biol. Chem. 78, 765.

VEIBEL, S. (1931), Über die Vorgänge bei der Phosphorylierung von Zucker durch Hefe, insbesondere über die Veränderungen während der Periode der totalen Phosphorylierung. Biochem. Z. 239, 350.

VENNESLAND, B., SOLOMON, A. K., BUCHANAN, J. M. and HASTINGS, A. B. (1942), Glycogen formation from glucose in the presence of radioactive carbon dioxide. J. biol. Chem. 142, 379.

VLÈS, F. (1926), Microcolorimètre pour les mesures microscopiques de pH ou de rH. C. R. Soc. Biol. Paris 94, 879.

VOGLER, K. G. (1942), Studies on the metabolism of autotrophic bacteria. II. The nature of the chemosynthetic reaction. *J. gen. Physiol.* 26, 103.

VORLÄNDER, D. und STRUBE, W. (1913), Die Einwirkung von Kohlensäure auf Alkalien und alkalische Erden, eine Zeitreaktion. *Ber. dtsch. chem. Ges.* 46, 172.

WALKER, H. H. (1932), Carbon dioxide as a factor affecting lag in bacterial growth. *Science* 76, 602.

WALLACE, W. M. and HASTINGS, A. B. (1942), The distribution of bicarbonate ion in mammalian muscle. *J. biol. Chem.* 144, 637.

WARBURG, E. J. (1922), Studies on carbonic acid compounds and hydrogen ion activities in blood and salt solutions. A contribution to the theory of the equation of Lawcnee J. Hendersson and K. A. Hasselbaleh. *Biochem. J.* 16, 153.

WARBURG, O. (1910), Über die Oxydationen in lebenden Zellen nach Versuchen im Siccigeli. *Hoppe-Seyl. Z.* 66, 305.

— (1934), Sauerstoffübertragende Fermente. *Naturwiss.* 22, 441.

— und CHRISTIAN, W. (1939), Isolierung und Kristallisation des Proteins des oxydierenden Gärungsferments. *Biochem. Z.* 303, 40.

WERKMAN, C. H. and WOOD, H. G. (1942), Heterotrophic assimilation of carbon dioxide. *Advances Enzymol* 2, 135.

WERTHEIMER, E. (1934), Über die ersten Anfänge der Zuckerassimilation. *Protoplasma* 21, 522.

WHERRY, W. B. and ERVIN, D. M. (1918), The necessity of carbon dioxide for the growth of *B. tuberculosis*. *J. infect. Dis.* 22, 194.

WHITE, B. (1938), The biology of *Pneumococcus*. New York. p. 421.

WILBRANDT, W. (1938), Die Permeabilität der Zelle. *Ergebn. Physiol. biol. Chem. exp. Pharmakol.* 40, 204.

WILLIAMS, J. W. (1939), Growth of microorganisms in shake cultures under increased oxygen and carbon dioxide tensions. *Growth* 3, 21. Cited in *Ber. ges. Physiol.* 117, 298 (1940).

WILLIAMS, R. J. (1920), A quantitative method for determination of vitamine. *J. biol. Chem.* 42, 259.

WILLMER, E. N. (1934), Some observations on the respiration of certain tropical fresh-water fishes. *J. exp. Biol.* 11, 283.

WILLSTÄTTER, R. und ROHDEWALD, M. (1937), Über die erste Phase der Gärung durch Hefe. *Hoppe-Seyl. Z.* 247, 269.

— und SCHUDEL, G. (1918), Bestimmung von Traubenzucker mit Hypojodit. *Ber. dtsch. chem. Ges.* 51, 780.

WILSON, G. S. (1931), The gaseous requirements of *Br. Abortus* (bovine type). *Brit. J. exp. Path.* 12, 88.

WINSLOW, C.-E. A., WALKER, H. H. and SUTERMEISTER, M. (1932), The influence of aeration and of sodium chloride upon the growth curve of bacteria in various media. *J. Bact.* 24, 185.

WOOD, H. G. and WERKMAN, C. H. (1935), The utilization of CO_2 by the propionic acid bacteria in the dissimilation of glycerol. *J. Bact.* 30, 332.

WOOD, H. G., and WERKMAN, C. H. (1936), The utilisation of CO₂ in the dissimilation of glycerol by the propionic acid bacteria. Biochem. J. 30, 48.

— and WERKMAN, C. H. (1938), The utilization of CO₂ by the propionic acid bacteria. Biochem. J. 32, 1262.

—, WERKMAN, C. H., HEMINGWAY, A. and NIER, A. O. (1940), Heavy carbon as a tracer in bacterial fixation of carbon dioxide. J. biol. Chem. 135, 789.

—, WERKMAN, C. H., HEMINGWAY, A. and NIER, A. O. (1941 a), Heavy carbon as a tracer in heterotrophic carbon dioxide assimilation. J. biol. Chem. 139, 365.

—, WERKMAN, C. H., HEMINGWAY, A. and NIER, A. O. (1941 b), The position of carbon dioxide carbon in succinic acid synthesized by heterotrophic bacteria. J. biol. Chem. 139, 377.

—, WERKMAN, C. H., HEMINGWAY, A. and NIER, A. O. (1941 c), Mechanism of fixation of carbon dioxide in the Krebs cycle. J. biol. Chem. 139, 483.

—, WERKMAN, C. H., HEMINGWAY, A. and NIER, A. O. (1942), Fixation of carbon dioxide by pigeon liver in the dissimilation of pyruvic acid. J. biol. Chem. 142, 31.

WOODS, D. D. (1936), Hydrogenlyases. IV. The synthesis of formic acid by bacteria. Biochem. J. 30, 515.

YUDKIN, J. (1932), Hydrogenlyases. II. Some factors concerned in the production of the enzymes. Biochem. J. 26, 1859.

ZECHMEISTER, L. und TÓTH, G. (1934), Über die Polyose der Hefemembran. I. Biochem. Z. 270, 309.

— und TÓTH, G. (1936), Über die Polyose der Hefemembran. II. Biochem. Z. 284, 133.

ZELLER, H. (1926), Wirkung von Ammonsalzen auf die Hefegärung. IV. Biochem. Z. 175, 135.

ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 10. SUPPLEMENTUM XXXI

FROM THE NOBEL INSTITUTE FOR NEUROPHYSIOLOGY,
KAROLINSKA INSTITUTET, STOCKHOLM

THE ACTION POTENTIAL
AND EXCITATORY EFFECTS
OF THE SMALL VENTRAL
ROOT FIBRES TO
SKELETAL MUSCLE

by

LARS LEKSELL

med. lic.

A T H E S I S

With the sanction of the Royal Caroline Institute submitted to public discussion in the Great Lecture Theatre of the Institute on May 17th 1945 at 9 o'clock a. m. for the acquirement of the degree of Doctor of Medicine.

STOCKHOLM 1945

To the memory of my father



CONTENTS

	page
Preface	7
Introduction	9
Methods	15
1. Experimental material	15
2. Amplifiers, electrodes	16
3. Recording devices	17
4. Stimulator	18
5. Devices for blocking nerve conduction	18
6. Myograph	19
Results	21
I. The action potential of the small fibres of the ventral roots	21
1. Form of the motor response volcley	21
2. The action potential in the ventral roots related to the histological picture	30
3. The conduction velocity of the small fibres of the ventral roots	33
4. Relative excitability of the two groups of fibres in the ventral roots	35
5. Differential block of conduction in the ventral root fibres	37
6. The action potential of the small ventral root fibres and the muscle action potential	40
II. Excitatory effects elicited by the small fibres of the ventral roots	44
1. The small fibres of the ventral roots and the muscle contraction	45

2. The small fibres of the ventral roots and the afferent discharge from the muscle	52
a) The efferent gamma fibres and the »backresponse» from the muscle.....	53
b) The efferent gamma fibres and the afferent activity from the muscle proprioceptives	59
Discussion	73
Summary.....	77
References.....	79

PREFACE

The present investigation has been carried out at the Nobel Institute for Neurophysiology where the author has had the privilege of working during periods of leave from his ordinary neurosurgical duties. It is thus an expression of the close contact maintained between this institute and the clinical departments.

To Professor RAGNAR GRANIT, Director of the Nobel Institute for Neurophysiology, I am indebted in many ways. It is a pleasant duty to express my gratitude to him for steady advice and encouragement during this work and for the truly inspiring time spent in his laboratory.

I also wish to take this opportunity to acknowledge my debt to Professor HERBERT OLIVECRONA, Director of the Neurosurgical Clinic, who has been my chief and teacher for many years, and whose interest and support has facilitated this work.

To Docent CARL GUSTAF BERNHARD and Docent CARL RUDOLF SKOGLUND my thanks are due for valuable help and for many stimulating discussions during these years.

I am particularly indebted to Mr. K. T. HELME, who has designed most of the apparatus used and given valuable instruction in technical matters.

To my wife, who has had a great share in this work, I wish to express my personal gratitude.

The investigation has been supported by grants to the Nobel Institute for Neurophysiology from the Rockefeller Foundation as well as by a grant from the Nobel Fund of the Caroline Institute, kindly voted by the Teacher's Collegium of this institute.

Stockholm, April 9th, 1945.

LARS LEKSELL

INTRODUCTION

The theme of this work is an analysis of the action potential and the functional significance of the group of small nerve fibres in the spinal ventral roots which innervate skeletal muscle.

It has long been known that the ventral roots of man and several other species in addition to the large motor fibres also contain numerous small fibres contributing to the sympathetic system (GASKELL 1886, Langley 1922, and others). In 1930 ECCLES and SHERRINGTON, however, showed that small fibres of the motor roots also pass down to the muscles. They studied the two last lumbar and the first sacral motor roots in the cat and found the myelinated fibres of these roots to be distributed in two distinct groups with numerical maximums at a diameter of approximately 15μ and 6μ respectively and separated by a minimum at about 8μ . A similar distribution of the efferent fibres was found in previously deafferented nerves to the muscles of the leg (see Fig. 1).

Sympathetic myelinated fibres in these nerves were few or totally lacking. Owing to the ramification of the fibres the average diameter of the efferent fibres was found to decrease towards the periphery, especially in the vicinity of the muscles, and in sections close to the muscle there was a partial filling out of the gap between the two fibre groups. Thus a number of the small fibres at distal levels were found to arise from large motor root fibres but ECCLES and SHERRINGTON concluded that the small fibres »for the most part emerged as such from the spinal motor root and are in fact small fibres of that root destined for the skeletal muscles».

Lately the small efferent fibres have been studied by especially HÄGGQVIST and his collaborators. In a series of papers the occurrence of such fibres in spinal motor roots and in different

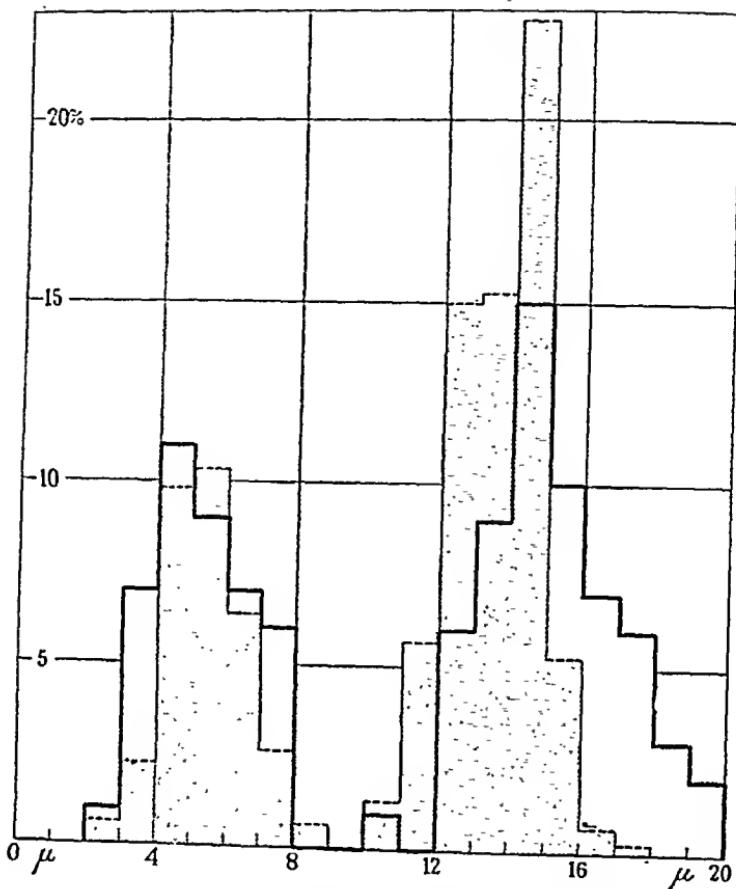


Fig. 1. Diagram of the fibre distribution of the 1st sacral motor root (—), and of previously deafferented nerve of gastroc. med. (---). From ECCLES and SHERRINGTON, *Proc. Roy. Soc., B.* 1930, 106, 326.

cranial nerves of man and other mammals has been carefully investigated (BJÖRKMAN and WOHLFART 1936, HÄGGQVIST 1937, 1938, SVENSSON 1938, REXED 1944). In the spinal roots the small fibres predominate in the thoracic and upper lumbar segments with their autonomic outflow but in most species there is a considerable number of fine fibres also in the roots to the muscles of the extremities. In man the small fibre group is insignificant in the lumbosacral but quite prominent in the cervical region (REXED 1944).

Physiological information about these small efferent fibres is scarce. In their pioneer work on the action potential of nerve

GASSER and ERLANGER as well as BISHOP with their collaborators have created the now well-known system of correlation between the component waves of the conducted action potential and the size of the fibres in the nerve responsible for them. Their work has mainly been devoted to somatic sensory nerves, large motor fibres and autonomic fibres. (ERLANGER and GASSER, 1924, ERLANGER, BISHOP and GASSER, 1926, GASSER and ERLANGER, 1927, ERLANGER, 1927, GASSER, 1928, BISHOP and HEINBECKER, 1930, ERLANGER and GASSER, 1930, HEINBECKER, O'LEARY and BISHOP, 1933, GASSER, 1935, ERLANGER, 1937, HURSH, 1939, GASSER and GRUNDFEST, 1939).

As to the large myelinated fibres, the A fibres, of mixed nerves, their component potential elevations, the alpha, beta, gamma and delta waves, have been identified in the posterior roots or in cutaneous sensory nerves and have been assigned to corresponding fibre groups. In the spinal motor roots, however, only an alpha potential has been observed. (ERLANGER, BISHOP and GASSER, 1926, ERLANGER and GASSER, 1930, O'LEARY, HEINBECKER and BISHOP 1934, ERLANGER 1937). The distribution of the different potential waves, hitherto observed in mixed nerve and in spinal roots, and their relative conduction rates are illustrated in the diagram of Fig. 2, reproduced from Erlanger (1937).

Of chief interest in this connection is the paper by O'LEARY, HEINBECKER and BISHOP (1934). On the basis of the work of ECCLES and SHERRINGTON they devoted a special study to the small efferent fibres and their functional significance. They confirmed the observations of ECCLES and SHERRINGTON concerning the distribution of efferent fibres of different size to the medial head of the gastrocnemius muscle in the cat. The action potential in excised lumbar and sacral ventral roots was found to show a typical A elevation with a maximum amplitude elicited by a stimulus approximately 3 times that of the most irritable fibres. A low tail is added to the main elevation by increasing the stimulus strength 4 to 6 times the threshold value». Conduction velocity in the fastest fibres

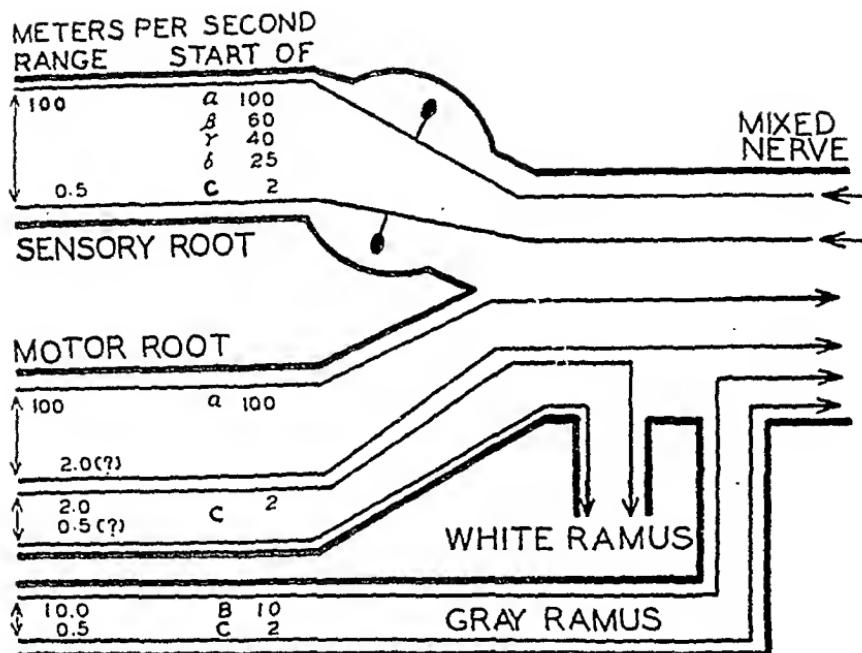


Fig. 2. Diagram of the distribution of the different potential waves in mixed nerve and spinal roots and their conduction velocity on the basis of a conduction rate of 100 in the largest *a*-fibre. From ERLANGER, in GASSER and ERLANGER, *Electrical Signs of nervous activity*. Univ. Penn. Press, Philadelphia, 1937.

was 110—80 m. p. s., the corresponding value for the fibres responsible for the tail of the potential could not be accurately measured but was estimated to be 15—20 m. p. s. In several roots a C potential was observed, in some preparations also a small B potential with a conduction velocity of 8—12 m. p. s. and a threshold 15—20 times higher than that for the fastest fibres. The action potential in both the normal and in the de-afferented nerve to the gastrocnemius showed a similar A potential with a low tail. The widening of the A potential at its base was held to be due to fibres »of the middle size range». No elevation that could have been ascribed to the group of small fibres was seen.

As to the functional significance of the small fibres different views are held. In the work mentioned above ECCLES and SHERRINGTON assumed them to have the same motor func-

tion as the large fibres but held them to innervate smaller motor units.

O'LEARY, HEINBECKER and BISHOP could not find a definite contractile effect ascribable to the small fibres. Maximal tension in the gastrocnemius muscle developed upon tetanic stimulation with a stimulus strength estimated to activate the large fibres alone. It could not be determined whether or not the small fibres elicited such a weak contraction that it was concealed by the effect of the large fibres. According to their view the small fibres may be concerned in the innervation of the intrafusal fibres of the muscle spindles. They were not able to demonstrate this directly, but support was found in DOGIEL's (1906) statement that muscle spindles are lacking in the eye muscles, supplemented by their own inference that the small efferent fibres also were absent in the oculomotor nerve. However, this argument is contradicted by BJÖRKMAN and WOHLFART (1936) who found the same fibre distribution in the abducens nerve of sheep and cattle as in man, cat and dog although muscle spindles are present in the former and absent in the latter species.

That the small fibres may innervate the muscle spindles is strongly suggested, however, by some findings by MATTHEWS (1933) in his fundamental study of the tension receptors in muscles of the cat. Working on single fibre preparations he isolated different types of nerve endings in the muscle. One of these types, the so called A 2 endings, exhibited a marked difference of behaviour during active isometric contraction of the muscle, depending on the strength of stimulus used. In most cases these endings began to discharge when the stimulus was supramaximal, and this fact makes it probable that they are innervated by smaller fibres than those responsible for the contraction of the muscle. According to MORSON and PHILLIPS (1937), however, the observation of MATTHEWS might possibly be due to an artificial rise of threshold of the axons to the intrafusal muscle fibres on account of ischemia and temperature changes of the nerve-preparation.

HÄGGQVIST has recently (1938, 1939, 1940) advocated the view that the small fibres constitute a separate motor system in the service of muscular »tonus». In histological studies on the innervation of the muscle fibres he came to the conclusion that the motor end organs of the type »terminaisons en grappe» were innervated by small fibres which in some cases could be traced backwards to the thin fibres in the muscle nerve. Fibre analysis of the nerves to muscles with »tonic» function, e. g. the *musculus sphincter ani ext.*, also showed a single group of small fibres.

In experiments with transitory ischemia of the spinal cord by temporary occlusion of the aorta in rabbits, HÄGGQVIST (1938, 1939) produced a spastic state in the hindlegs of the animals. Fibre analysis of the ventral roots in these animals then showed a selective degeneration of the large fibres whereas the small fibres were practically intact. As in these experiments only very few fibres belonging to the group of large fibres were left, he concluded that the small fibres were responsible for the spasticity of the muscles. Hypertonic states have also been produced by VAN HARREVELD and MARMONT (1939) with the aid of spinal cord ischemia caused by increasing intradural pressure, a method already used by CUSHING 1902.

The first aim of the present work was to identify and analyse the action potential wave corresponding to the small fibres and to study some of the electrophysiological properties of these fibres. This part of the work is described in the first section of the paper. In section II further experiments are found dealing with the effect of the small fibres on the muscular contraction and on the afferent discharge from the muscle.

METHODS

1. Experimental material.

Cats have been used in the experiments. Generally they were anaesthetized with Dial »Ciba» 0.5 ml. per kg. intraperitoneally. Sometimes an intravenous injection of 1 % chloralose, 5 ml. per kg., was given instead. In other experiments decerebrated animals were used. The experiment then was begun some 2—3 hours after the decerebration, performed in ether narcosis in the usual manner.

In a limited number of experiments frogs were used. This preparation consisted of the sciatic nerve excised together with the 9th or 10th motor root. The preparation was placed in a moist chamber of plexiglass and investigated at room temperature (18—20° C).

The experiments on cats were carried out with nerves *in situ*, always the sciatic or one of its branches together with one of its ventral roots, L 6, L 7 or S 1. After lumbar laminectomy the roots were ligated and cut centrally and placed alongside the cord until taken up to be placed on electrodes. In preparing the nerve, care was taken to avoid interfering with the circulation. When it was desired to study the effect in the muscle the gastrocnemius was used. This too was dissected free with special attention devoted to preserving its circulation intact. The other muscles of the thigh and the calf were denervated. An aluminium wire attached to the Achilles tendon, connected the muscle with the myograph, the leg itself having been fixed with drills through the lower ends of femur and tibia. Further details are given in connection with the experiments to be described.

The animal was mounted in a screened box with the aid of clamps fixed to the spinous processes. The box was electric-

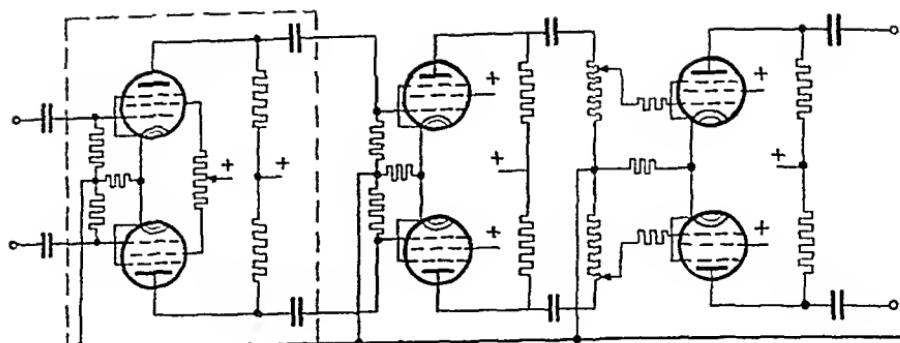


Fig. 3. Circuit diagram of amplifier.

ally heated to 35—37° C, and a moist atmosphere for the nerve was secured by a heated water bath. These precautions kept the rectal temperature of the animal at about 37—38° C.

2. Amplifiers, electrodes.

Two resistance-capacity coupled amplifiers were employed. The one, chiefly used for the recording of the action potentials, contained three pentode stages, push-pull coupled throughout. The two last stages belonged to a Philips cathode ray oscillograph type G. M. 3156. This oscillograph has an asymmetrical input which for these experiments was adapted to a symmetrical initial stage with Philips C F 50 pentodes, heated from the mains. The circuit diagram of the amplifier is found in Fig. 3. The time constant is 0.17 sec. and the frequency curve is shown in Fig. 4 a. A calibration record at maximal amplification is found in Fig. 4 b.

The second amplifier was a similar 3-stage push-pull coupled unit combined with a power stage for a loudspeaker.

The recording and stimulating electrodes have been silver-silver-chloride wires ending in a cotton wick with an interelectrode distance of about 1 cm.

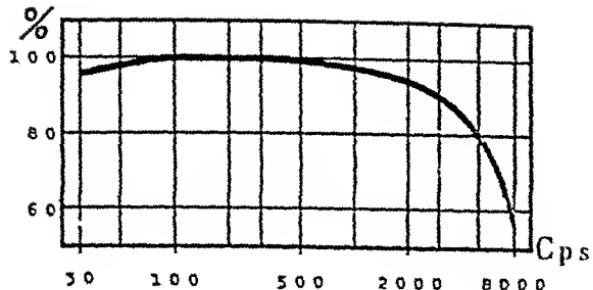


Fig. 4 a.

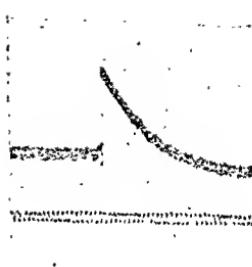


Fig. 4 b.

Fig. 4 a. Frequency curve of the amplifier.

Fig. 4 b. Calibration of the amplifier at maximal amplification. Input 30 mV. Time 1/50 sec.

3. Recording devices.

Two cathode ray tubes have been used, the one a Philips type DB 9 with a single ray, the other one an AEG type HR 2 with double rays. Either amplifier could be connected to any of the three available beams. The third beam was projected onto the film by means of a mirror.

The sweeps of the two oscillograph aggregates were started by means of a contact which also, after a delay of a couple of milliseconds elicited the stimulus. The contact was closed by means of an automatically working relay, starting the sweep at a slow frequency of about 0.5—2 per sec. In some cases the sweep was tripped directly by the stimulus. The beams of the cathode ray oseillograph were swept horizontally across the film which was run at a very slow speed, at about 1.5 cm. per sec., so that the individual beats of the sweep appeared well separated. The time, marking the film speed, was 0.2 sec.

As time marker for the sweep served a Philips frequency generator, type G. M. 2307 which also was used for the recording of the muscle contraction by a method of modulation to be described below. The time is always in milliseconds, unless otherwise mentioned.

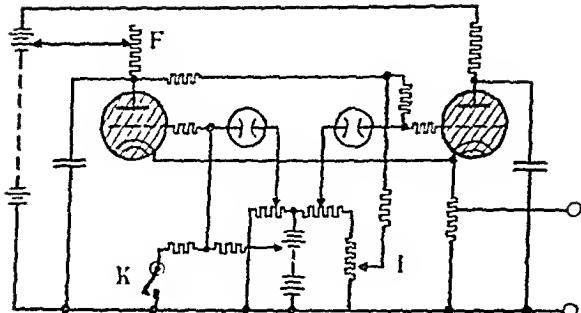


Fig. 5.

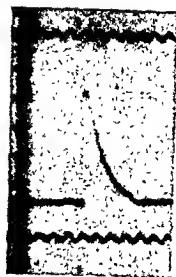


Fig. 6.

Fig. 5. Circuit diagram of the stimulator.

Fig. 6. Form of stimulus shock. Time 0,1 msec.

4. Stimulator.

Thyatron controlled condenser shocks served as stimuli. They were discharged over a transformer. In Fig. 5 is found the circuit diagram of the stimulator. In order to obtain two successive shocks at variable time intervals two thyatron tubes with a common cathode resistance were used. The first shock was started by the contact *K*. It was followed by the second shock at an interval which could be regulated by altering the grid potential by means of the potentiometer *I*. For single shocks only the first tube was used. When iterative stimuli were desired, as *e. g.* for tetanus, the grid potential of the tube was regulated in such a manner that it worked automatically at a frequency, controlled by the anode resistance *F*. The form of the stimulus shock is shown in Fig. 6.

5. Devices for blocking nerve conduction.

In order to differentiate between the effect of large and small nerve fibres selective blocking of the large fibres was tried by means of two methods: pressure block and block by direct current. The pressure block was first produced by means of a small pneumatic pressure chamber. It was found, however, that an equally selective block could be achieved by a simple

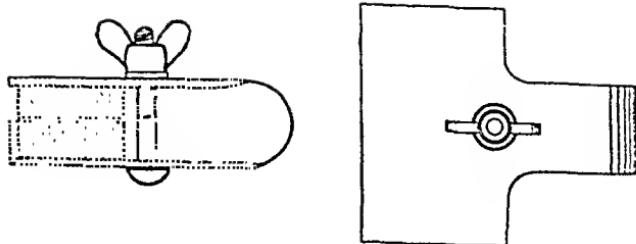


Fig. 7. Clamp for nerve compression. $\frac{1}{1}$.

spring clamp, padded with soft rubber cushions and tightened with a screw (Fig. 7). In some experiments pressure was applied by the simple expedient of compressing the nerve for some 15—20 minutes between the thumb and the forefinger.

For the polarization block electrodes of the silver-silverchloride type were used and the current taken from a battery over a potentiometer. Resistances in series with the nerve and a large shunting condenser facilitated an even increase of the current when the sliding resistance of the potentiometer was shifted.

6. Myograph.

For the recording of the muscle contraction by one of the cathode rays a myograph, utilizing changes of mutual inductance between two movable coils, was designed by the physicist of this laboratory, Mr K. T. HELME.

To the lever of a torsion-wire myograph was attached a small coil mounted perpendicularly to another fixed coil. The one coil was fed with sinus waves at a frequency of 8 000—10 000 c. p. s. from the Philips frequency generator, the other was coupled to the input of a simple 3-stage R. C.-amplifier. The mutual inductance alters with every angular change of the two coils relative to one another so that, when the muscle pulls on the myograph, and in consequence thereof the angle between the coils alters, the amplitude of the induced sinus current increases. This change is recorded by the

amplifier. In its last stage one phase of the sinus current is suppressed by suitable adjustment of the grid potential and the other phase is ultimately recorded by the cathode ray as an area, the upper contour of which reproduces myograph tension (see *e. g.* Figs. 24 and 32).

By altering the position of the coils relative to one another or by adjusting the volume control it is easy to regulate the sensitivity of the instrument from a maximum deflection of 2 mm. per 10 gm. downwards. By varying the grid potential of the last valve by means of a potentiometer it is possible to make the myograph deflection practically linear with respect to myograph tension or, alternatively, to record the top or the bottom of the isometric curve with a proportionately greater sensitivity. The myograph was calibrated by loading it with weights or, during the actual experiment, by testing with a spring balance.

RESULTS

I

The action potential of the small fibres of the ventral roots

1. Form of the motor response volley.

In their experiments on the action potentials of motor fibres ERLANGER, BISHOP and GASSER, already in 1926, used the mammalian sciatic nerve with one of its roots. This preparation has also been used here, partly because it is possible to obtain a relatively long conduction distance and partly because the fibre distribution in the muscle nerves as well as in the spinal roots is known from the work of ECCLES and SHERRINGTON (1930).

In some preliminary experiments the frog nerve was studied. The stimulus was applied at the distal end of the sciatic nerve and the action potential was recorded from the 9th or 10th ventral root. If the conduction distance is long enough the well-known alpha wave is succeeded by a separate little action

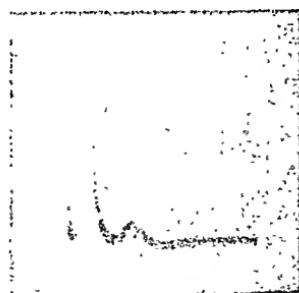


Fig. 8. Action potential of 9th ventral root of frog in response to stimulation of the sciatic nerve.

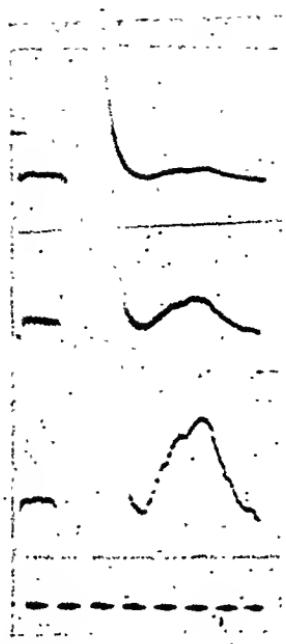


Fig. 9. Action potential in the ventral root L 7 of cat in response to stimulation of the sciatic nerve. In the uppermost record relatively low amplification; in the following records the amplification has been successively increased up to maximum.

potential wave. This has a longer latency and a higher threshold than the alpha wave. In Fig. 8 is illustrated the result of one of these early experiments on the frog sciatic. The technique had not yet been developed to the stage used in the main series. However, the wavelet is clearly visible. At the amplification used, a large fraction of the alpha wave falls outside the screen of the cathode ray.

A similar wave was seen in cats and in the main work these animals were used alone because they were more suitable for the experiments on the muscle. The sciatic stem or one of its branches together with the motor roots L 6, L 7 or S 1 were studied *in situ*. Granted a sufficient conduction distance, there is a separate second wave also in these records of which Fig. 9 provides a sample. The form and size of this small elevation varies a great deal from case to case. In some experiments it has been difficult to find, or has even been totally lacking. In

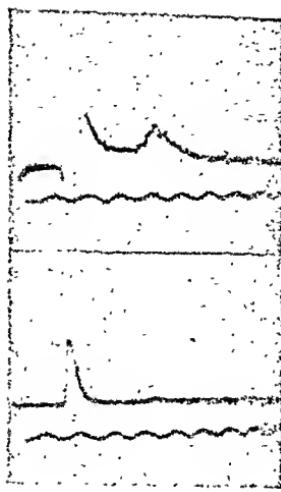
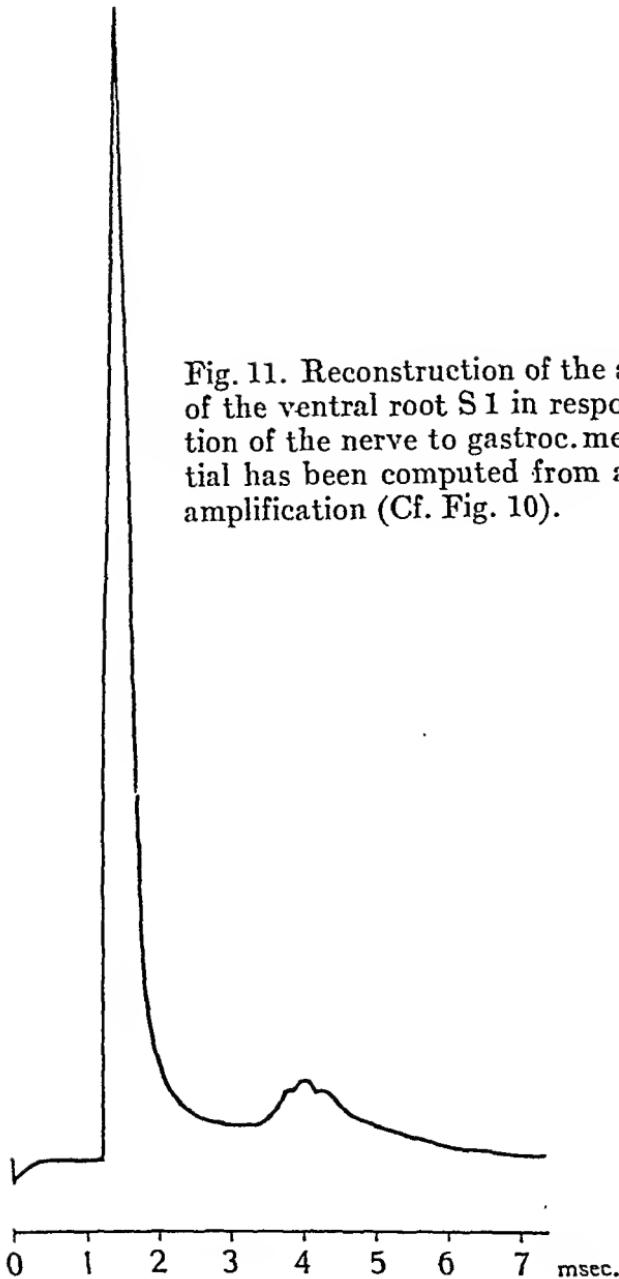


Fig. 10. Action potential in the ventral root S 1 in response to stimulation of the nerve to gastroc. med. The amplification in the upper record 9 times greater than in the lower record.

most experiments, however, it could be clearly observed. Fig. 9 refers to stimulation at the distal end of the sciatic with leads on L 7. In the uppermost record amplification is moderate and, consequently, the small wave merely a hump on the baseline. In the two following records amplification has been increased in order to define the small wave more clearly. The alpha wave falls largely outside the screen of the cathode ray. Its amplitude is approximately 15 times that of the small wave. Another experiment is shown in Fig. 10. In this case the nerve to the medial head of gastrocnemius was stimulated, and records were taken from the first sacral ventral root. In the lower record the alpha potential was photographed at low amplification; in the uppermost record the amplification was 9 times greater and consequently the second wave stands out clearly. In Fig. 11 a reconstruction has been made, showing the whole wave of action. Its alpha component has been computed from records at low amplification.

If the discharge is sent in the opposite direction it is possible to obtain a similar picture. In general, however, the second wave does not stand out well in records from peripheral nerve.

Fig. 11. Reconstruction of the action potential of the ventral root S 1 in response to stimulation of the nerve to gastroc. med. The α -potential has been computed from a record at low amplification (Cf. Fig. 10).



Probably the fibres concerned are shunted by the large number of sensory fibres. Other factors, too, may have contributed to the occlusion of this wave. In Fig. 12 the 1st sacral root has been stimulated and the leads have been on the medial nerve to gastrocnemius. In this particular case the small wave is clearly visible.

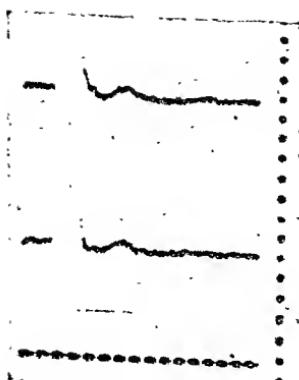


Fig. 12. Action potential in the nerve to gastroc. med. in response to stimulation of the ventral root S 1.

It seemed reasonable to ascribe the second small wave of action to a separate group of small high-threshold fibres. Nevertheless sources of error are not excluded and should be given due consideration. One alternative would be iteration of activity in the large fibres, another that a similar picture might be due to subdivision of the fibres.

It is possible to show, however, that the small wave cannot be due to iterative activity in the large fibres. If the conduction distance is long the large and the small wave are well separated from each other but when the distal stimulating electrode is shifted upwards along the nerve the small wave approaches the large wave and finally is completely submerged in the latter. In Fig. 13 *a* and *b* the leads have been on L 7 and the stimulating electrodes on the proximal part on the sciatic at distances of approximately 10 and 7 cm. from the leads. At these relatively short distances the small wave is merely a hump at the base of the alpha wave.

Further arguments against identification of the small wave with an iterated alpha potential have been obtained from the experiments on selective blocking of the motor fibres. It is possible to block the large potential wave almost completely without interfering to any considerable degree with the small wave. Again, when iterative activity in the motor fibres is observed it is generally, though not always, of a very

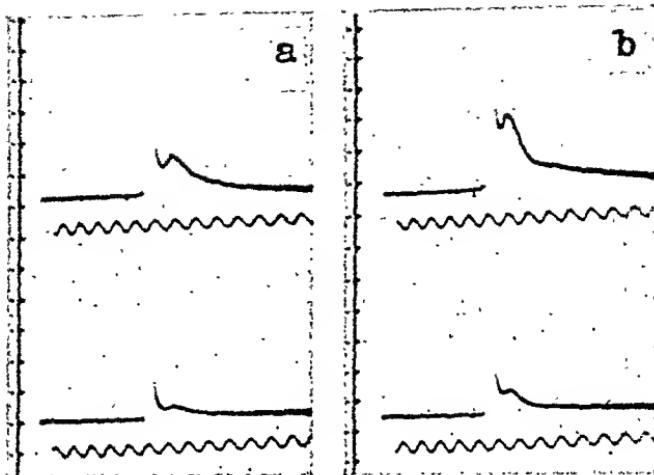


Fig. 13. Action potential of the ventral root L7 in response to stimulation of the sciatic nerve. *a*. Conduction distance 10 cm. *b*. Conduction distance 7 cm. Amplification in the upper records 3 times greater than in the lower set of records.

characteristic pattern, illustrated in Fig. 14. In this record there are several successively diminishing wavelets. The animal had been narcotized with chloralose, a drug tending to favour iterative activity. The leads were on the ventral root S 1, the stimulating electrodes on the popliteal branch of the sciatic. In Fig. 14 *a* there is also, between the alpha wave and the first iterated response, a couple of wavelets which under certain circumstances may complicate evaluation of the small wave studied in this paper. These wavelets are centripetal discharges in the motor fibres originating at the muscle nerve endings (LLOYD 1942). This »back-response» from the muscle, which is further dealt with in the next section of the paper, disappears when the nerve is severed above the muscle (Fig. 14 *b*) and so can hardly be mistaken for the small wave found above.

The small wave cannot either be due to subdivision of the larger fibres. Its high threshold does not fit in with this explanation of its origin. The threshold is considerably higher than that of the alpha wave and, by increasing stimulus strength gradually in the upper ranges, it is also possible to augment the small wave gradually. If this wave was due to branching

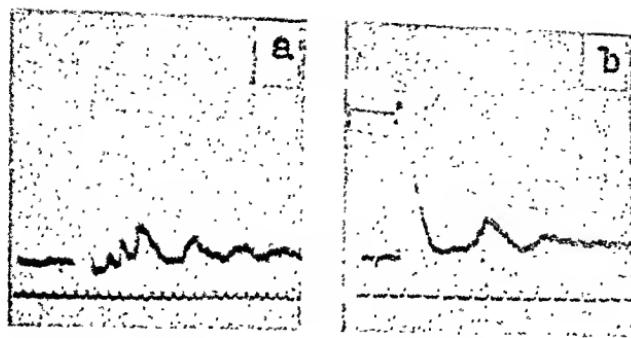


Fig. 14. Repetitive activity in the ventral root S1 in response to stimulation of the popliteal nerve.

a. Stimulation of undivided nerve.

b. After section of the nerve peripherally to the stimulated region.
Note! Different sweep velocities.

of the larger fibres the two waves should appear together at approximately the same strength of stimulus. In none of these experiments with centripetal conduction of the action potential could any signs of branching be observed. When leading off in the periphery, however, as e.g. from the medial gastrocnemius nerve, a long drawn-out tail of the alpha potential is often seen (Cf. Fig. 23). It has approximately the same threshold as the alpha fibres and is probably due to branching of fibres. As ECCLES and SHERRINGTON have shown, the large fibres divide distally and the gap between the two fibre groups is then partly filled out. This low-threshold tail of the alpha wave can hardly be mistaken for the second small wave. It is possible, however, that some branching of the fibres contributes to the difficulties encountered in recording the small wave from the peripheral end of the nerve.

In order to prove the separate origin of the small wave it was finally decided to try the following crucial experiments, modelled on those of ERLANGER and GASSER, and by means of which they demonstrated the individuality of the different potential elevations in mixed nerve. (ERLANGER and GASSER, 1924, 1930.)

In the experiment of Fig. 15 double stimuli, first, conditioning, and second, test stimulus, have been applied to the tibial

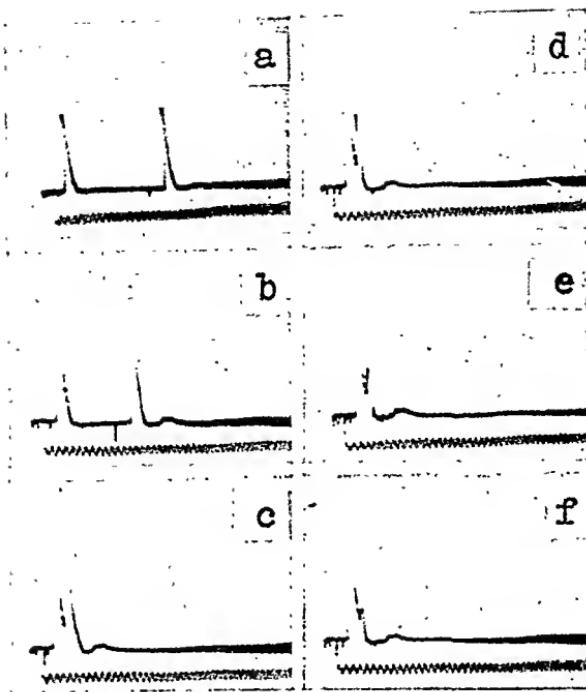


Fig. 15. Records showing that the fibres conducting the alpha wave can be made refractory without affecting the small wave. The first stimulus maximal for alpha, the second maximal for the small wave. In record *a* relatively low amplification. In the following records higher amplification and successively shortened stimulus intervals. See text.

nerve and the leads have been on the motor S 1. The first stimulus is maximal for alpha but below the threshold for the small wave, the second stimulus is strong enough also to elicit the small wave for which it is just supramaximal. The interval between the stimuli shortens successively so that the second alpha wave gradually falls within the refractory period of the first. Then its amplitude diminishes (Fig. 15 *c-f*) and finally this second alpha wave altogether disappears. The small wave, however, is still intact, showing that it is conducted in a different set of fibres, not engaged by the alpha response.

In the experiment of Fig. 16 the first stimulus is supramaximal for the small wave, the second, however, only max-

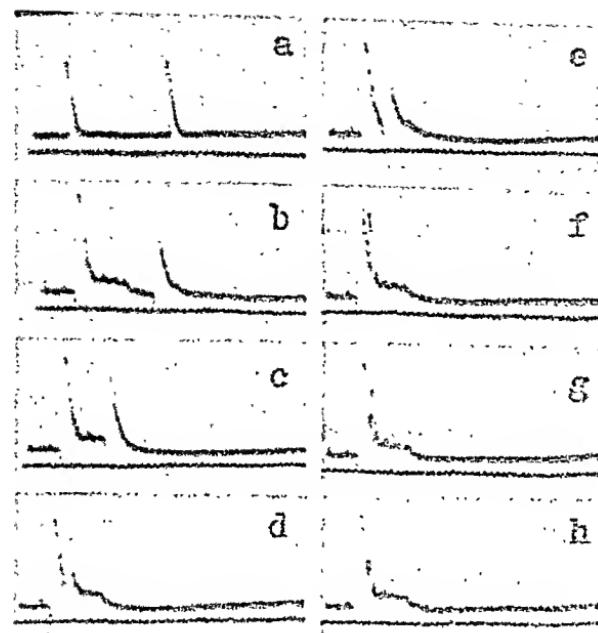


Fig. 16. Records demonstrating that an alpha wave can be made to pass the small wave during conduction without influencing the form of the latter. In *a* low amplification. In the following records greater amplification and stimulus intervals successively diminished. See text.

imal for the initial alpha wave but subthreshold for the small wave. The tibial nerve was stimulated and the leads were on the motor S 1. The small wave in this case is not very well set off from the alpha wave but nevertheless distinguishable. Again stimulus interval is shortened. The second alpha wave is then seen to approach the first and to pass the small wave (Fig. 16 *c—e*). Ultimately it falls into the refractory period of the first alpha wave and disappears. It is thus seen that the second alpha wave reaches the leading-off electrodes at the same time as, or somewhat before, the small wave though the latter started later. The second alpha wave has thus, in being conducted upwards, on account of its higher conduction rate, passed the small wave. The small wave, however, is uninfluenced by these events.

From these two experiments it may be definitely concluded that the small wave is conducted in fibres which do not conduct the large alpha wave. It must thus be due to a separate group of fibres.

2. The action potential in the ventral roots related to the histological picture.

After these experiments it seems probable that the small wave is due to action potentials in the well-known group of small fibres of diameters from $3-8 \mu$. This conclusion has been checked with the aid of a comparison of the time relations of the small wave with the histological picture in a manner, well known from the classical work of GASSER and ER-LANGER (1927).

It has now been shown by GASSER and his collaborators (HURSH, 1939, GASSER and GRUNDFEST, 1939) that the conduction velocity of the nerve fibres is approximately proportional to their diameter, not to the square of the diameter as at one time was believed. GASSER and GRUNDFEST showed that the conduction velocity stands in this relation to the axone diameter. In agreement with ARNELL (1936) they found that the ratio between axone diameter and outside diameter is not constant within the whole fibre range. For fibres below 8μ the ratio successively sinks from 0.69 to 0.55 at 3μ , so that the speed of conduction within this range diminishes at a faster rate than that corresponding to the diminution of the outside diameter.

In comparing the conduction velocity of the action potential with fibre calibre the following procedure was adopted. The action potential was recorded as monophasically as possible and at great amplification, so that the small wave became clearly visible. The small wave was then copied under a magnifying projector. Below the time scale was plotted the calibre scale, assuming that the largest fibres initiating the alpha wave had a diameter of 20μ (see Fig. 1). The calibre scale was

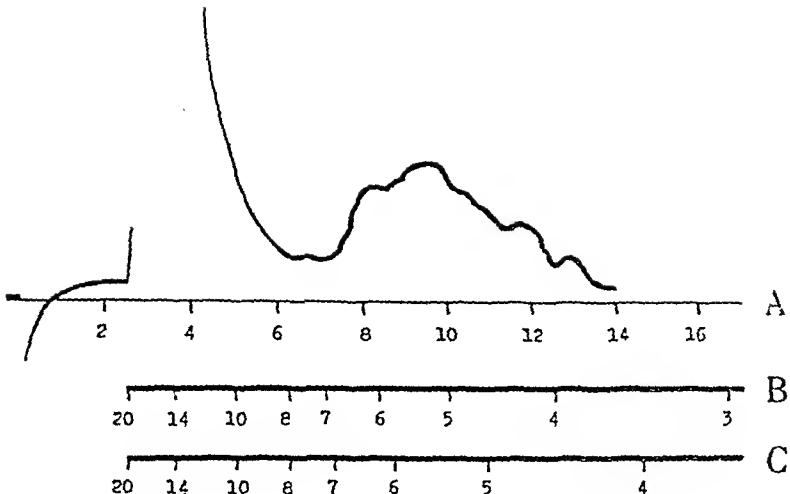


Fig. 17. Correlation between the action potential and the size of the ventral root fibres. The action potential was recorded in the ventral root S 1 in response to stimulation of the tibial nerve. A, time scale in msec. B and C, calibre scales of the outside diameters of the nerve fibres in μ . Scale B corresponds to a direct relation between outside diameter and conduction velocity. Scale C corresponds to a direct relation between axone diameter and conduction velocity. See text.

plotted in two ways: (i) on the assumption of a direct proportionality between conduction velocity and outside diameter for the whole range of fibre diameters down to 3μ , (ii) the same scale was corrected for the small fibres so as to agree with the sinking ratio between axone diameters and outside diameters.¹ Thus this particular scale corresponds to a linear relation between conduction velocity and axone diameter.

In Fig. 17 has been pictured the action potential in S 1 elicited by stimulation of the tibial nerve. It is seen that the calibre scale which corresponds to direct proportionality between conduction velocity and axone diameter (Scale C) is spread out over a wider range than the uncorrected scale re-

¹ According to GASSER and GRUNDFEST's curve for this ratio the values for 7, 6, 5 and 4μ respectively are approximately 0.68, 0.66, 0.63 and 0.59. Hence the value in this scale relative to the time axis will be, for instance, for 5μ $0.63/0.68$ of the value in the scale corresponding to a linear relation between conduction velocity and outside diameter.

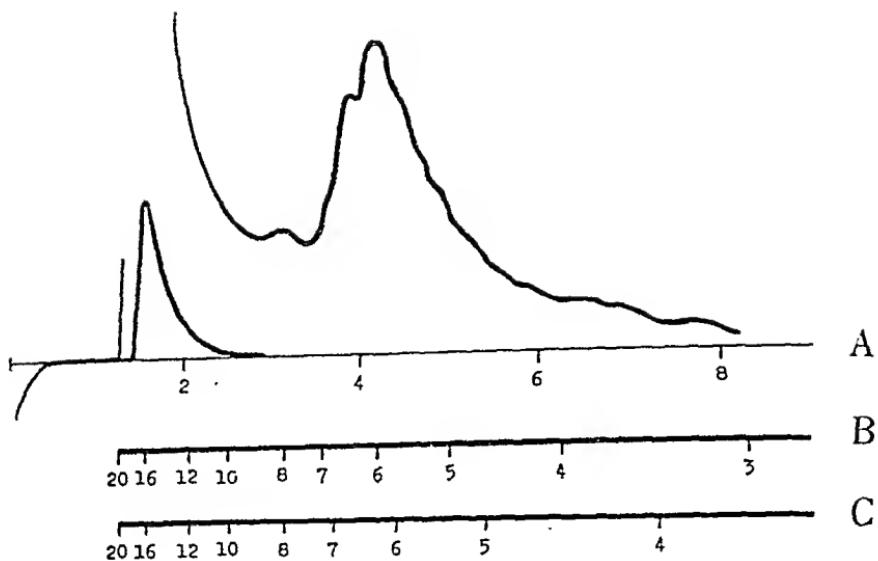


Fig. 18. Correlation between action potential and fibre size as in fig. 17. The action potential was recorded in the ventral root S1 in response to stimulation of the nerve to gastroc. med. The action potential of the second wave recorded at an amplification 27 times greater than that used for recording the α -potential in full. See text.

ferring to outside diameter (Scale B). But this difference is of significance only for calibres below 5—6 μ .

A glance at the figure shows that the minimum of the curve of potential corresponds to a fibre size between 7 and 8 μ . This corresponds well with the histologically demonstrated minimum between the two fibre groups. The maximum of the second wave is between 5 and 6 μ . The end point of the potential cannot be given with precision since the wave of action ends in a flat, extended tail. On the scale, based on direct proportionality between axone diameter and conduction velocity (Scale C), the potential runs down to about 4 μ .

In Fig. 18 is shown another example of the relation between calibre and action potential. The medial nerve to gastrocnemius was stimulated and the leads were on the ventral S1. In addition the alpha wave is reproduced in full at an amplification that is smaller than the one used for the small wave.

Here too the minimum is around 8μ . The peak of the alpha wave corresponds to a diameter of about 16μ . The maximum for the small wave falls around 6μ and the wave is followed down to $3-4 \mu$.

A comparison between fibre distribution, according to ECCLES and SHERRINGTON (see Fig. 1), and action potential thus shows very good agreement between the distribution of potential, on the one hand, and the distribution of fibre size on the other. The spike potential allotted to the small fibres is found in the right place in the diagram and, no doubt, is also caused by activity in the group of small fibres.

3. The conduction velocity of the small fibres of the ventral roots.

The conduction velocity of the largest motor fibres which correspond to the spikes making up the initial phase of the alpha potential is well known from several investigations. It is practically the same as for the largest sensory fibres, with values around 100 m. p. s. O'LEARY, HEINBECKER and BISHOP (1934) found rates varying between 80 and 110 m. p. s.

With these figures available it was held unnecessary to measure the absolute value for conduction velocity in the small fibres in separate experiments at different conduction distances. As a rule only their relative conduction rates were determined. The conduction velocity of the alpha wave was given the value of 100 and that of the small wave computed from the records in relation to this fixed value. Measurements on the records were made from the shock artefact to the start point of the alpha wave and to the start and the end point of the small wave. The ventral roots were again used for the leads and the stimulus was placed at some peripheral nerve, *e.g.* on the distal part of the sciatic. A relatively high degree of amplification was used so that the wave stood out clearly. Stimulus strength was maximum for the small wave.

The values obtained in 5 experiments have been collected

Table 1. *Relative conduction velocities of ventral root fibres.*

Stimulated nerve	Recorded ventral root	Start of alpha-wave	Start of second wave	End of second wave
Sciatic	L 7	100	33	20
Sciatic	L 7	100	40	20
Peroneal	L 7	100	38	21
Gastroc. med. . . .	S 1	100	41	25
Tibial	S 1	100	38	21

in Table 1 in which the conduction velocity of the alpha wave is fixed at 100.

The conduction velocity of the largest fibres of the small fibre group is thus, on an average, 38 per cent of that of the largest alpha fibres. The lower limit can not be very accurately measured, because the potential wave ends in a flat, extended tail. The conduction velocity in the small fibres, however, ranges down to approximately 20 per cent of the alpha rate.

In two experiments the absolute value of the conduction velocity of the small wave was determined. In the one experiment, with leads on the ventral root S 1 and stimulation of the distal part of the tibial nerve, the conduction distance was 23 cm. The conduction velocities for the alpha wave and the small wave were respectively 92 m. p. s. and 33 m. p. s. In the other experiment, with leads at S 1 and stimulation of the medial gastrocnemius nerve, the conduction velocities were respectively 115 m. p. s. and 44 m. p. s. for the large and the small fibres.

If the values thus obtained for the small efferent fibres are compared with the conduction velocities of the well-known A components in sensory nerves it is found that the nearest equivalent to these efferent fibres is the sensory gamma group.

Referring back to ERLANGER'S diagram, reproduced on p. 12 it is seen that, relative to an alpha velocity of 100 m. p. s., the sensory gamma wave is conducted at a rate of 40 m. p. s., i. e. at a speed closely corresponding to that of the small efferent

fibres. For this reason, and in order to have a convenient term for »the small potential wave» and »the small fibres of the ventral roots» they will below be called the »efferent gamma» wave and the »efferent gamma» fibres.

4. Relative excitability of the two groups of fibres in the ventral roots.

It has often been pointed out that the relative irritabilities of fibres of different conduction velocities are functions of the time constants of the stimulus. Thus the greater excitability of the coarse fibers is less prominent with stimulating currents of long duration (ERLANGER, 1937, BISHOP and O'LEARY, 1939). With the common rapid condenser discharges, used also in this work, it has, however, been estimated (O'LEARY, HEIN-BECKER and BISHOP, 1934) that fibres below 8μ require at least $2\frac{1}{2}$ times stronger stimuli than fibres around 20μ .

The relative irritabilities of the two fibre groups have been determined from simultaneous records of action potential and stimulus on the double ray oscillograph. The relation between stimulus strength and the recorded deflection was so adjusted, that the latter had a suitable amplitude on the face of the tube where it was directly measured. Strength of stimulus was measured for (i) alpha wave threshold, (ii) alpha maximum, (iii) gamma wave threshold, (iv) gamma maximum.

Maximum strength for the gamma wave was held to have been reached at a strength of stimulus at which the tail of this wave was fully developed. Amplification was adjusted so as to reproduce the gamma potential in full but was diminished temporarily in order to measure the amplitude of alpha.

The results from five experiments have been summarized in Table 2.

It should be noted that the definition of the alpha maximum does not exactly correspond to the definition of the gamma maximum since in the former case maximum amplitude is used and in the latter case it was required that the tail of the gamma wave also be visible. The reason for this difference in the de-

Table 2. *Relative thresholds of ventral root fibres.*

Stimulated nerve	Recorded ventral root	Alpha threshold	Alpha maximum	Gamma threshold	Gamma maximum
Sciatic	L 6	1	3.3	3.7	13
Peroneal	L 6	1	3.5	3.5	15
Peroneal	L 7	1	3.1	3.9	23
Gastroc. mcd. . . .	S 1	1	3.3	4.0	12
Tibial	S 1	1	2.3	4.2	16

definition of the maximum is the great dispersion of the gamma wave which means that, on increasing stimulus strength, this wave expands horizontally rather than vertically.

Table 2 shows that the threshold of the most excitable units within the group of small fibres are, on an average, 3.9 times higher than those of the most sensitive coarse fibres. Related to the alpha threshold as unity a 16fold increase of stimulus strength is necessary for activating the whole group of small fibres. Even though all figures must be regarded as round numbers there is, nevertheless, a considerable difference in the irritability of the two groups of fibres. As a rule there is a distinct interval in stimulus strength between the maximum for alpha fibres and the absolute threshold for the gamma fibres. The difference in stimulus strength between the late basal portion of the alpha wave and the initial phase of the gamma wave is insignificant but, at times, clearly measurable.

Thus, at a stimulus strength necessary for including the whole alpha complex there is never more than a small fraction of the gamma fibres engaged, judging from the small area of the gamma potential present at this strength of stimulation. A successive increase of stimulus strength will therefore engage all fibres of the alpha group before a significant part of the gamma fibres has become mobilized. These facts are of primary importance for any attempts to isolate the functional properties of the two groups of fibres.

5. Differential block of conduction in the ventral root fibres.

In order to investigate the function of the efferent gamma fibres it is desirable to be able to eliminate the activity of the large fibres. To this end experiments were carried out with pressure block and polarization block. The selectivity of the block was tested with the action potential as index.

GASSER and ERLANGER showed in 1929 that the pressure block first attacked the coarse fibres whereas cocaine first paralysed the small fibres. Since that time such methods of differentiation have been used a great deal in investigations on man and animals, chiefly in order to study the function of various sensory fibre groups. (For work on animals, see CLARK, HUGHES and GASSER, 1935, BISHOP and HEINBECKER, 1935.) The mechanism of the block and its dependence upon pressure and ischemia has recently been studied by BENTLEY and SCHLAPP (1943). The constant current block has been much less used and is, according to BISHOP, HEINBECKER and O'LEARY (1933), somewhat less selective than the pressure block. Cocaine too, in primarily attacking the small fibres, is less selective than pressure (GASSER and ERLANGER 1929).

The pressure block was applied to the sciatic nerve with the metal clamp described above (p. 19), or, simply, by pressing the nerve with the fingers. The stimulus was applied to the peripheral end of the nerve and the leads were on the motor roots. It proved possible to obtain a highly selective block by these means. The large main wave could be removed without any marked effect on the small gamma wave. The degree of pressure applied has been varied so that a total block of the alpha fibres was developed within a time varying between 15 and 60 min. Within wide limits the degree of selectivity is independent of the amount of pressure exerted, but, as it was felt desirable to reach the full effect before marked changes in the general experimental conditions had occurred, relatively heavy pressure was used. Possibly the selectivity of the block is also somewhat more pronounced at high pressures. Often the

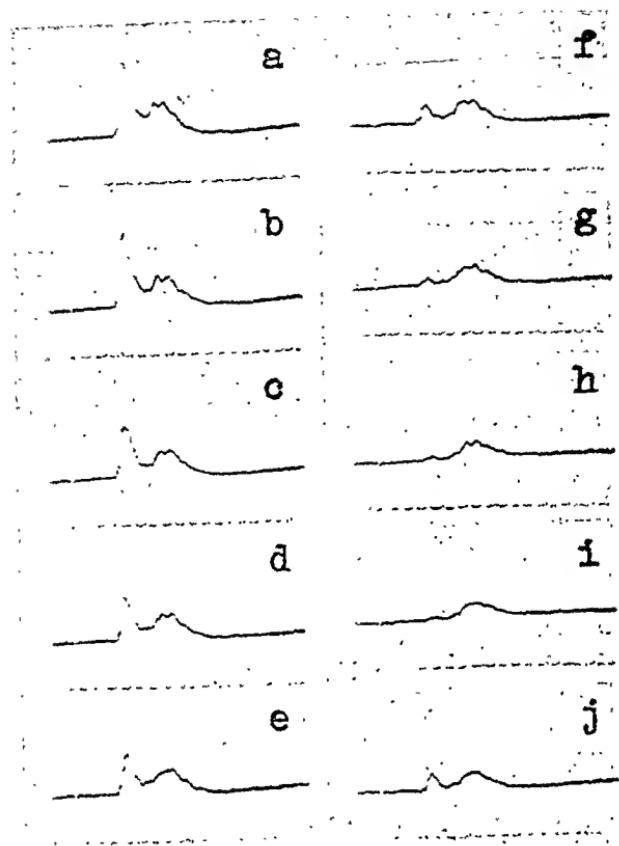


Fig. 19. Differential blocking of the α -fibres by compression. The potential is recorded from the ventral root S1 in response to stimulation of the nerve to gastroc. med. Block applied to the sciatic nerve. The records show the final stages of the experiment approximately 30 min. after application of pressure.

best result was obtained if pressure first was exerted till the alpha wave had disappeared, then removed for a brief interval, and finally applied again after some rotation of the nerve so that the fibres were struck in a new place. In Fig. 19 are shown the last stages of an experiment in which pressure was applied manually for 25 min. In Fig. 19 *a* the size of the alpha potential is already considerably reduced. Its amplitude is only about $1/10$ of the initial height. The gamma wave, however, is still of normal size.

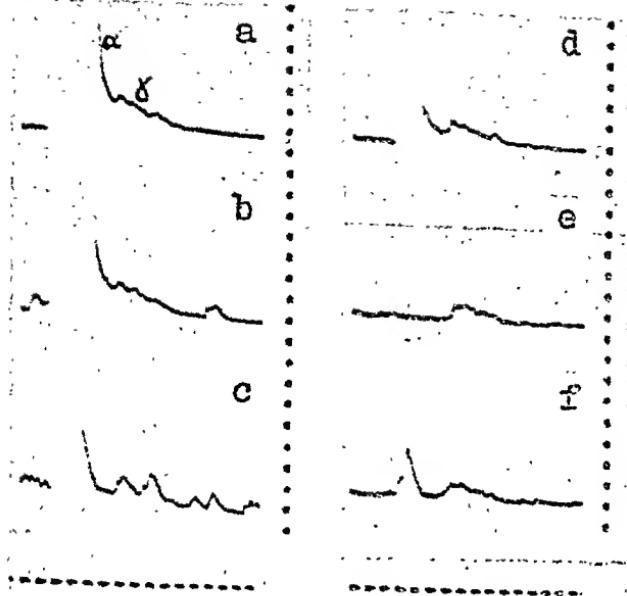


Fig. 20. Differential blocking of the α -fibres by polarization. The action potential was recorded from the ventral root S 1 in response to stimulation of the nerve to gastroc. med. Blocking electrodes at the sciatic nerve with the cathode distally. Current strength is successively increased from *a* to *e*. In *f* polarizing current broken. Current strength in *c* approximately 0.5 mA.

In Fig. 19 *b* to *g* the block proceeds and the alpha wave is further reduced. Only a very slight diminution of the gamma wave takes place, however, and finally a stage is reached when merely gamma potential is left (Fig. 19 *i*). In Fig. 19 *j* pressure has been removed and then a small alpha wave has turned up again.

It is important to pay attention to the possibility of iterative discharges emanating from the blocked region. (Cf. SCHAEFER and SCHMITZ, 1933). As a rule such iterative discharges in the motor fibres, whether spontaneous or elicited by the arrival of the impulse to the blocked region, have not been noted in these experiments.

In the experiments with polarization block the current has been applied to the sciatic nerve. The best selectivity is

obtained with the cathode towards the stimulus. Then the polarization block may be just as selective as the pressure block. This is shown in Fig. 20. In this particular experiment the gamma wave was included in the tail of the alpha potential (Fig. 20 a). But upon increase of strength (Fig. 20 b—e) the alpha components successively disappear leaving the gamma wave practically intact (Fig. 20 e). In a certain phase of the block there was a heavy repetitive discharge from the blocked region (Fig. 20 c). This, however, disappeared upon further augmentation of current strength.

In a few experiments the cocaine block was tried. Ethocaine 1: 500 was sucked up in a piece of cotton wool that was wrapped around the nerve. It was found that the effect upon the gamma wave was greater than the effect on the alpha wave but, since the small fibres easily can be eliminated by utilizing the threshold difference, it was held unnecessary to use this less selective method of removing them.

Compared with the pressure block the polarization block has the advantage that it can be applied easily and is repeatable. But the repetitive discharge leading to muscle tetanus in a certain phase of the advancing block is a disadvantage when the effect of the small fibres upon the muscle activity is under investigation. For this reason pressure block has been the chief method of removing the alpha component of the nerve action potential.

6. The action potential of the small ventral root fibres and the muscle action potential.

In view of the two waves of the motor response in the sciatic it is necessary to ask whether it is possible to demonstrate a similar subdivision of the muscle action potential. It would not seem unreasonable to assume that the small fibres activate muscular structures giving some separate kind of electrical response.

Actually several authors have reported that two components can be seen in the muscle spike, *e. g.*, GÖPFERT and SCHAEFER

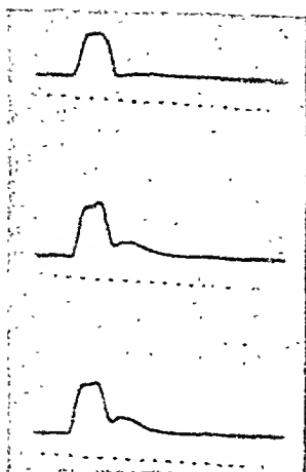


Fig. 21. Action potential of the gastrocnemius muscle, recorded between the cut end and the belly of the muscle. Stimulus strength is successively increased downwards.

(1941), ROSENBLUETH, WILLS and HOAGLAND (1941), WILLS (1942). This subdivision of the muscle action potential has been ascribed to a subdivision of the muscle into fast and slow fibres but has not been correlated with the wave of action in the nerve which hitherto has been regarded as merely consisting of the relatively homogeneous alpha wave.

In order to investigate this problem the action potential of the gastrocnemius muscle was rerecorded with the intention of revealing a possible muscular index of activity in the gamma fibres.

The stimulating electrodes were on the sciatic nerve. In order to make the muscle potential as monophasic as possible the distal electrode was placed on a tendon beyond a crushed region or on a muscle cross section. The proximal electrode was on the belly of the muscle. Other muscles were denervated.

The recorded action potentials varied a great deal in form, depending upon the localization of the electrodes. Quite often the potential was divided in two parts, a large initial spike and a second small wave more or less distinctly separated from the former, as in Fig. 21. The small wave often had a higher threshold than the large spike.

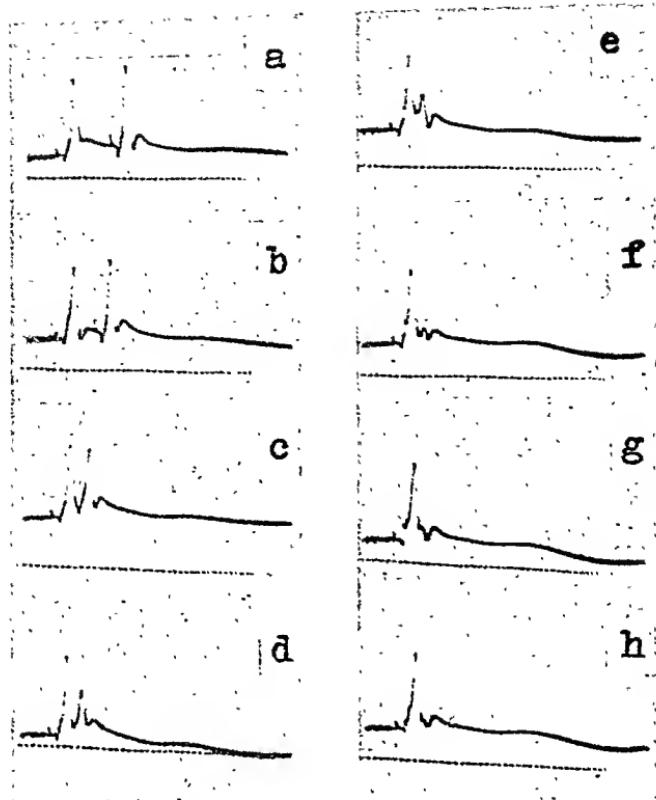


Fig. 22. Records showing that the muscle fibres which produce the first large spike of the muscle action potential can be made refractory without affecting the fibres producing the second small wave. See text.

In some cases the second wave must be ascribed to the diphasic artefact (Cf. BISHOP and GILSON, 1927, 1929). However, it is impossible to explain all records in this way. For its independent nature also speak results obtained in experiments similar to those described above for the nerve potential. An experiment of this kind is illustrated in Fig. 22.

Two successive stimuli were applied to the sciatic nerve. The first stimulus was nearly maximal for the first action potential spike, the second stimulus strong enough to be maximal also for the small wave of the complex potential. (Fig. 22 *a, b*). At decreasing intervals the second large wave gradually fell into the refractory period of the first (*a-c*), diminished (*d-f*) and

disappeared (*g—h*). The small wave, however, was uninfluenced. It cannot therefore have originated in the same elements as the large spike.

From this experiment it seems probable that there are two independent elements in the muscle but it is not possible to infer that the second element is activated by the group of small gamma fibres in the nerve. It may just as well have been activated by smaller fibres of the large fibre group. In order to clarify this matter, further experiments seem desirable. However, it was felt that an analysis of the functional significance of the small gamma fibres of the motor nerve by means of muscle potentials introduced an element of hazard that made this approach both uncertain and tedious. The muscle potentials vary too much in form and secondary waves of different types turn up for reasons altogether beyond experimental control. It was therefore decided to employ other methods of analysis.

II

Excitatory effects elicited by the small fibres of the ventral roots

The identification of the action potential that corresponds to activity in the small fibres of the motor roots has facilitated the study of the excitatory effects elicited by these fibres. Systematic investigations have been devoted to the effect of the gamma fibres on the muscle contraction and on the afferent impulses discharged from the muscle. In these investigations it has been possible to utilize both the threshold differences between the alpha and gamma fibres as well as their differential sensitivity to pressure and polarization blocks. In both ways, the one procedure often supported by the simultaneous use of the other, isolation of the activity of the two fibre groups could be achieved.

The effects have generally been investigated in the gastrocnemius muscle or in soleus and gastrocnemius together. The muscle tension has been recorded isometrically and the afferent activity has been studied in the medial or lateral nerve from the gastrocnemius muscle. It was pointed out above that, as a rule, the gamma potential cannot be observed in records from peripheral nerve. Consequently it has not been possible to picture the gamma wave in the nerve to the muscle and use it as an index of the activity of the small fibres. Therefore, in some of the experiments, as a check on the block, a centripetal volley was sent through the blocked region and the gamma-potential was led off from a ventral root. In other cases the alpha potential was recorded peripherally and used as index of gamma activity and stimulus strength, it being known from the previous experiments that the gamma fibres are not activated

before the alpha wave has reached maximum. Thus an approximate control of the gamma activity in the nerves to the muscle was attained.

1. The small fibres of the ventral roots and the muscle contraction.

O'LEARY, HEINBECKER and BISHOP (1934) concluded from their experiments that the fibres of small calibre were without a definite effect on the muscle contraction. The maximum tension was reached at a stimulus strength $2\frac{1}{2}$ times the alpha threshold. Further increase of stimulus strength of up to 8—10 times the threshold did not augment the muscle contraction.

A number of experiments of this type correlating stimulus strength and muscle contraction were carried out one of which is illustrated in Fig. 23. The stimulating electrodes were on the ventral L 7, and the contraction of the gastrocnemius muscle — single twitches — was recorded along the left side of the film without use of the sweep circuit. As a check on stimulus strength and gamma activity the alpha potential was led off from the medial nerve to the gastrocnemius. The nerve was divided just above the muscle and the action potential recorded monophasically.

In Fig. 23 *a* stimulus strength is submaximal. In record *b* the alpha wave is nearly maximal. The mechanogram shows that the contraction too almost has reached its maximum. In the records *c* stimulus strength was successively increased (*1* to *3*) so that it ultimately reached a value about 5 times the one needed for maximum alpha. The mechanogram, however, shows but a negligible increase of the amplitude of the contraction. It is by no means certain that this small increase is due to gamma activity. It may just as well have been caused by a small number of high threshold alpha fibres.

Similar experiments with tetanic contractions instead of twitches gave identical results. Increase of stimulus strength beyond that necessary for maximum alpha did not augment

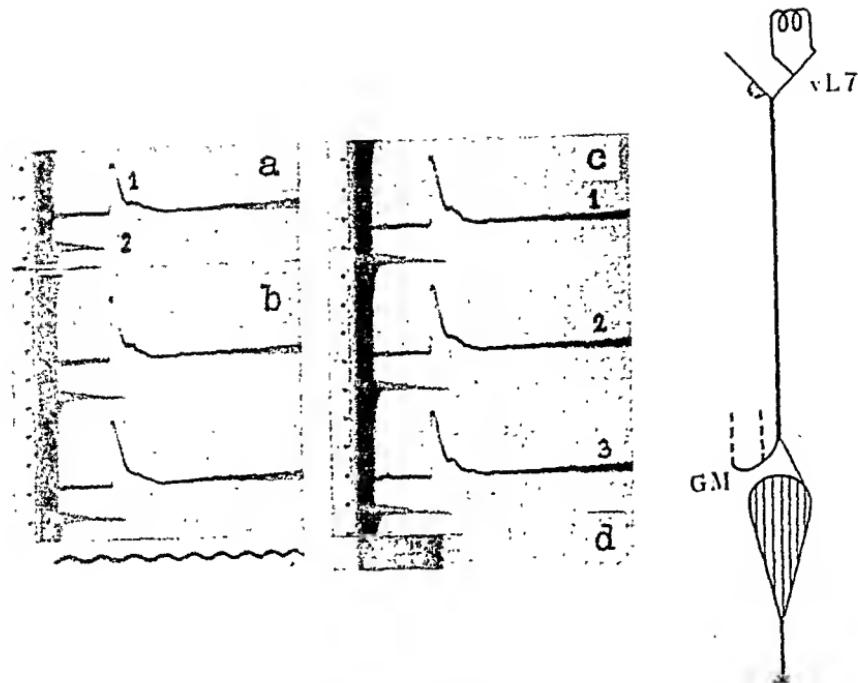


Fig. 23. Isometric twitch in gastrocnemius in response to stimulation of ventral root L7 (v L7 in diagram) with successively increasing stimulus strength. Simultaneous recording of the α -potential in gastroc. med. (GM in diagram).

Record *a*. Stimulus submaximal. 1, α -potential in gastroc. med. 2, muscle twitch.

b. Stimulus just submaximal for α .

c. Stimulus strength successively increased to approximately 15 times α threshold (1—3).

d. Calibration of myograph to 2 000 gm.

(The low-threshold tail of the α -wave is presumably due to branching of fibres. Cf. p. 27).

Full description in text.

the muscle tension. If a minimal muscle contraction was used as a measure of the threshold effect the maximum tension was found to correspond to 2.2—2.5 times the threshold value. In Fig. 24 is reproduced an experiment in which muscle tension and stimulus strength have been simultaneously recorded.

The stimulating electrodes were applied to the sciatic nerve, about 4 cm. above the gastrocnemius head. A low film speed was used and the sweep not employed so that the directly re-

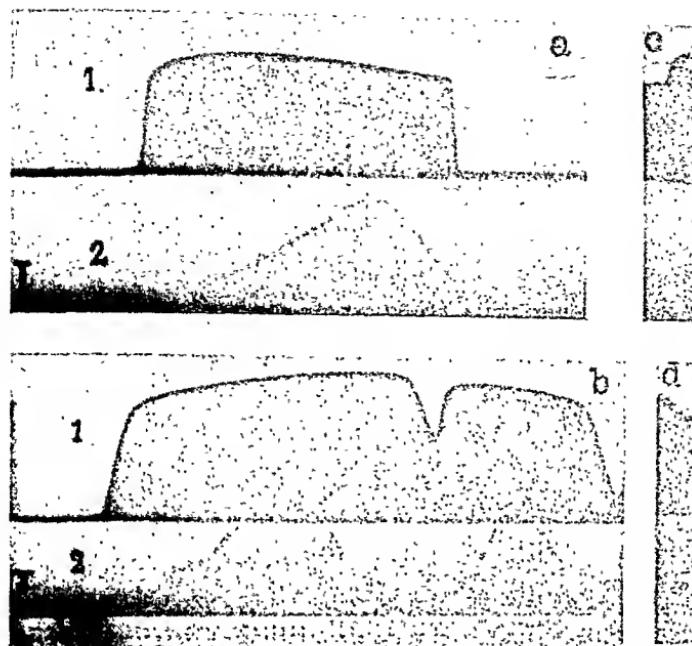


Fig. 24. Isometric tetanus in gastroenemius in response to stimulation of the sciatic nerve with varying stimulus strengths.
 a. 1, mechanogram. 2, relative stimulus voltage. Height of oblong T = threshold stimulus.
 b. Same as a. Time 1/5 sec.
 c. Myograph calibration for record a 1 2 000 and 2 250 gm.
 d. Myograph calibration for record b 1 2 250 and 2 000 gm.
 Full description in text.

corded stimulus shocks (*a* 2) form a surface the upper contour of which marks relative stimulation voltages. In order to obtain an accurate value for the threshold contraction this was first determined at maximal sensitivity of the myograph. A stimulus strength corresponding to a contraction of 20 gm was taken to be the threshold value (*T*). This having been done, the amplification of the myograph amplifier was increased, and the muscle contraction recorded in the manner illustrated in Fig. 24 *a* 1 and *b* 1. In these records the sensitivity of the myograph system was adjusted so that it was relatively greater at maximal than at minimal amplitude of the contraction, a

possibility provided for by the particular myograph system used (Cf. section on Methods). The successive increase in stimulus strength may be followed in the records. At approximately 2.5 times the threshold (T) the contraction was maximal. Further increase of up to 12 times the threshold strength had no effect on muscle tension. In *b* is shown, once more, that augmentation of stimulus strength above approximately 2.5 times the threshold did not affect the amplitude of the muscular response.

These results therefore agree with those of O'LEARY, HEINBECKER and BISHOP. If the gamma fibres have any effect on the muscle contraction this must be so small as to be unmeasurable, at least as long as the alpha fibres maintain full activity.

In drawing conclusions from these results one factor should be considered, on account of which this type of experiment cannot be regarded as crucial. This is the possibility of a multiple innervation of the muscle fibres by both alpha and gamma components. If there be multiple innervation, the results of these experiments as well as of those of O'LEARY *et al.* could be misleading.

Now multiple innervation has been described by several histologists (AGDUHR, 1916, 1919, 1939, CUAJUNCO, 1932, HÄGGQVIST, 1940, and others).¹ HÄGGQVIST (1940) has reported that muscle fibres in *m. tensor fasciae latae* in rabbits are innervated both by a coarse and a thin fibre. A physiological demonstration of multiple innervation has recently been given by KATZ and KUFFLER (1941). This factor cannot therefore be neglected. If the same muscle fibres are innervated by both alpha and gamma nerve fibres, it is quite possible for the effect caused by alpha activity to conceal whatever additional effects might have ensued from the high threshold gamma activity.

In order to eliminate the alpha activity and to find out whether isolated gamma fibres could elicit contractions, it was deemed necessary to carry out experiments with pressure block.

¹ For histological data concerning the innervation of skeletal muscle the reader is referred to the excellent review by HINSEY (1934).

The stimulating electrodes were applied to the ventral roots L 7 or S 1, and the muscle contraction recorded as in the experiment of Fig. 23. The pressure block was applied to the sciatic nerve. Its effect was checked by means of records of the alpha wave in the peroneal nerve or by recording the antidromic volley, sent alternately from below the block to a ventral root adjacent to the one stimulated, *e. g.* L 6. In the former case the progress of the block could be ascertained from the height of the alpha spike. In the latter case the blocking effect on both alpha and gamma fibres could be followed in the root when a number of sweeps were photographed in which alternately the muscle contraction and the antidromically elicited root potential were recorded.

Fig. 25 illustrates an experiment with single muscle twitches elicited from L 7, the block being checked by alternating between recording the mechanogram and the antidromically conducted volley in L 6. The records show the last stage of the blocking effect and the muscle contraction has been rerecorded at maximal myograph sensitivity. They were photographed between the 14th and 20th minute after application of a heavy pressure block. In Fig. 25 *a* there are first two beats of the sweep, 1 and 2, which show that the effect of the pressure has been to reduce the amplitude of the alpha wave in the ventral L 6, originally 15 times larger than the gamma wave, to only 3 times the gamma wave. The latter is practically intact. After the second beat of the sweep (2) the stimulus was switched over from the peroneal nerve to the ventral root L 7 in order to record the muscle contraction (M) which is of a very small order, in fact, 80 gm tension as against the original 500 gm before the block was applied.

The block has undergone further development in Fig. 25 *b*, and in *c* the amplitude of the remaining alpha spike roughly corresponds to that of the gamma wave, the latter still being relatively good. The rerecorded muscle tension is now below 50 gm. Finally, in *d*, there is a very small remnant left of the alpha spike. The gamma spike has also diminished but an increased amplification, as in the lowest beat of the sweep (*a* and *y*),

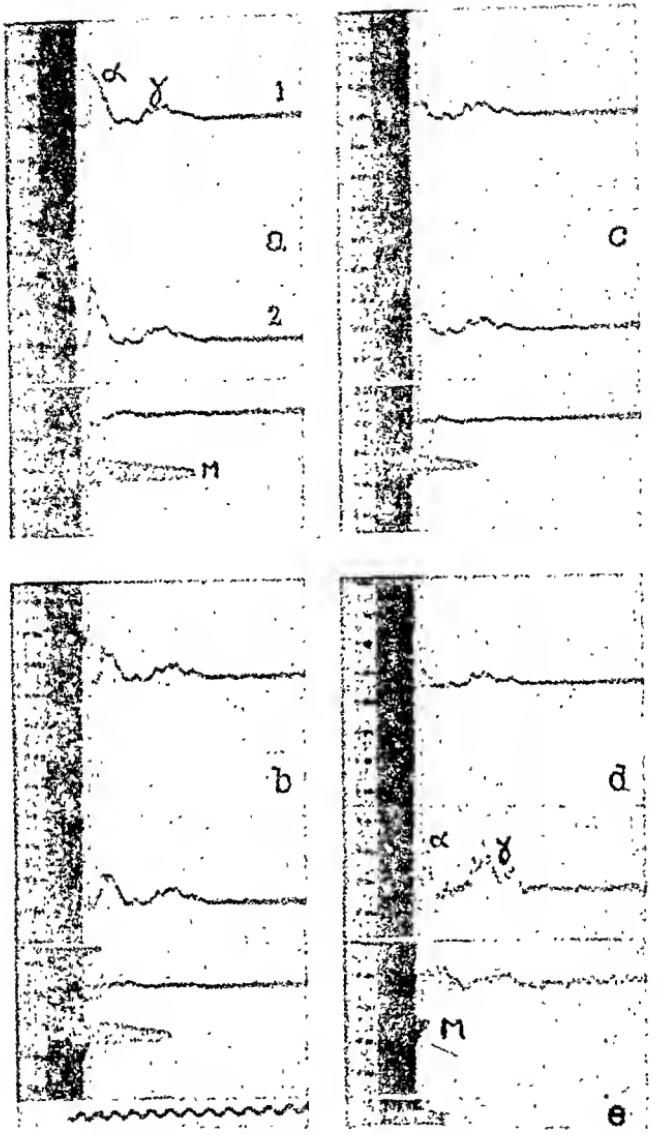


Fig. 25. Muscle twitch and differential blocking of conduction in α fibres. Isometric twitch elicited in gastrocnemius by stimulation of ventral root L 7 (v L 7 in diagram). Compression block on the sciatic nerve at B. Block controlled by alternating antidromic volleys from peroneal nerve, P, to v L 6. Initial stretch 200 gm. Initial twiteh 500 gm.

Records: a. Blocking time 14 min. 1 and 2, antidromic volleys in v L 6. M, the corresponding muscle twitch after switching over stimulus to v L 7. Twiteh tension 80 gm. (The sweep beat above

shows that its area still is considerable and a great deal larger than the area covered by the alpha wave. There is now merely a contraction of about 10 gm tension, *i. e.* 2 per cent of the original value.

The block developed in a quite satisfactory manner, as shown by the control of the antidromic volley recorded from L 6. Hence it must be concluded that, at the moment when the block had reached the stage that a very small, barely recordable fraction of the muscle contraction was left, a large fraction of the gamma population of fibres were still conducting through the blocked region. A very small number of alpha fibres were also capable of carrying impulses through the block and these or the gamma fibres must account for the minute effect of 10 gm tension recorded.

Similar results were obtained with tetanic stimulation. This is clearly shown by the experiment illustrated in Fig. 32, p. 68, 69. It was designed to elucidate the possible effect of gamma activity on the afferent discharge from the muscle but it also gives some information about the effect of a progressing block on a tetanic muscle contraction. The ventral root L 7 was stimulated supramaximally for gamma and the effect of the block was checked by means of records of the peroneal alpha potential. The muscle contraction was recorded without use of the sweep circuit and thus appears alongside the film on the left.

In Fig. 32, *c* and *d*, the block has reduced the muscle contraction to merely about 15 gm tension. At the same time the alpha spike in the peroneal nerve (*P*) has been considerably reduced.

the mechanogram is the record from v L 6 to stimulation of v L 7 and only shows shock artefact).

b. After 15 min.: twitch tension 60 gm.

c. After 17 min.: twitch tension 46 gm.

d. After 20 min.: twitch tension *M* approximately 10 gm. Amplification for the second sweep (*a, γ*) 3 times higher than in the preceding records.

e. Calibration of myograph to 20 gm.

Full description in text.

Nevertheless it is still clearly visible, and, with due regard to the selective properties of the block, it may safely be concluded that the majority of the gamma fibres are intact. Their effect on the muscle contraction, however, is minimal. This is now merely about 1 per cent of its original size which was 2 000 gm. This small remaining contractile effect may just as well be ascribed to the few remaining alpha fibres as to the gamma fibres.

It has thus not been possible to demonstrate any definite effect of the gamma fibres upon the muscle contraction. These last mentioned experiments should have removed the objection that was based upon the possibility of multiple innervation with consequent concealment of the gamma activity by the large contractile effect on the part of the alpha fibres. All such statements should, of course, be qualified by a reference to instrumental sensitivity. It is clear, however, that the limiting factor has been the selectivity of the block rather than the sensitivity of the myograph. The minimal contractions recorded have been of the order of 10—20 gm or about 2 per cent of the maximum tension. At this final stage of the blocking effect some alpha fibres have still remained active together with a very much larger number of gamma fibres. Since the former are known to be true motor fibres they are probably responsible for the contractile remainder. A minor contractile effect may be caused by gamma fibres. This effect, however, if it exists at all, is so small that it could not play any significant part in the muscular contraction regarded as a process for conversion of energy into movement and tension.

2. The small fibres of the ventral roots and the afferent discharge from the muscle.

It has not been possible to demonstrate any significant contribution on the part of the small efferent fibres to the muscle contraction. The next step in an investigation aiming at an understanding of their physiological task necessarily involves the afferent side. Is there any connection between the discharge in

the efferent gamma fibres and the afferent impulses from the muscles?

Until quite recently the afferent impulses from the skeletal muscles were all ascribed to various sensory end organs, the discharges of which had been well analyzed by MATTHEWS (1931, 1933). MATTHEWS also discussed the possibility of direct electrical stimulation of the afferent system by the action potential of the intrafusal muscle fibres but found evidence for this view only in one preparation. In eserized preparations recurrent activity in ventral roots was observed by DUN and FENG (1940) and MASLAND and WIGTON (1940).

Later LLOYD (1941, 1942) found that synchronous stimulation of a ventral root actually caused a centripetal discharge from the muscle. Unaware of his work I made the same observation¹ and then found that my records confirmed his in every detail. My results followed directly from the technical arrangements used in order to elucidate the problems raised by the responses in the gamma fibres. The stimulating electrodes were on the motor root and the leading-off electrodes on the sensory root of the same segment. Then the electrical «backresponse» from the muscle, in response to the efferent volley, turned up in the sensory root, before the afferent discharge from the muscle and tendon organs. LLOYD's work suggests that the electrical field around the intramuscular nerve endings initiates this centripetal discharge. Clearly this effect complicates the question raised and has to be dealt with first.

a. The efferent gamma fibres and the «backresponse» from the muscle.

The cross-excitation leading to the electrical backresponse from the muscle is found not only in sensory but also in motor fibres. When leading off the gamma potential from a ventral root the backresponse may sometimes appear simultaneously

¹ On account of the war it has been difficult to keep up contact with American physiological literature. Lately, however, several journals have arrived in this country.

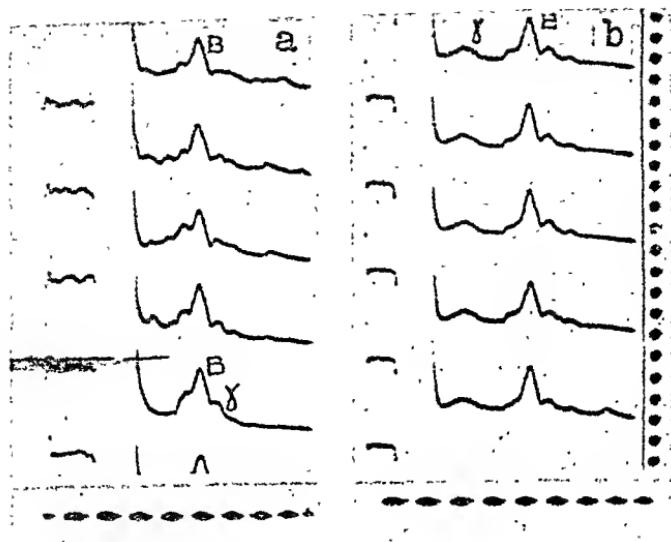


Fig. 26. Simultaneous appearance of »backresponse» and gamma wave in a ventral root. Records from ventral L 7 in response to stimulation of the undivided sciatic nerve.

a. Stimulation of the nerve 2,5 cm. above the gastrocnemius muscle. In the uppermost record the alpha wave is followed by a backresponse from the fore-leg muscles (*B*). In the following records stimulus strength is increased and a gamma wave appears (*γ*) with approximately the same latency as the backresponse.

b. Stimulation of the sciatic nerve 5 cm. more proximally than in *a*. The latency of the gamma wave (*γ*) is shortened, that of the backresponse (*B*) is lengthened.

with the gamma wave and complicate the evaluation of the record. An example will make this clear.

In the experiment in Fig. 26 the potential in the ventral root L 7 is recorded upon stimulation of the undivided sciatic stem, 2,5 cm above the gastrocnemius muscle. The experimental conditions are then favourable for giving both backresponse and gamma wave practically identical latencies. In the uppermost beat of the sweep circuit the large alpha wave is succeeded by a second irregular potential wave (*B*) that is the backresponse from the leg muscles. The latent period of this wave is about 4 msec. Strength of stimulus has been just subthreshold for the gamma

potential. In the following records of the same series *a*, the stimulus strength has been successively increased. The gamma wave does not appear as a separate entity but it is seen that the second potential wave has become elevated suggesting that the gamma wave (γ) has contributed something to this deflection of the instrument. By shifting the position of the stimulating electrodes upwards along the nerve this surmise was proved to have been correct. The effect of this shift was to shorten conduction distance for the gamma wave and lengthen conduction distance for the backresponse by the same amount. Accordingly the latent period for the gamma wave became shortened and that of the backresponse lengthened. This is illustrated in Fig. 26 *b* where the gamma wave (γ) turns up in front of the backresponse (*B*). The experiment presented in Fig. 26 illustrates that the backresponse, unless recognized, may seriously endanger interpretation of the gamma wave.

In the sensory roots the impulses from the muscles are far more marked. The discharge from a single muscle, *e.g.* the gastrocnemius, is of characteristic form: a synchronized volley with a tail of irregular shape. Assuming the gamma fibres to innervate separate elements, the tail might be a backresponse caused by the action potentials of these elements. In Fig. 27 is found the centripetal volley in the dorsal root L 7 after stimulation of the ventral L 7. The second complex potential (*2* and *3*) is coming from the gastrocnemius, the first (*1*) from proximal hip muscles that have been left undenervated. The response from the gastrocnemius is seen to consist of a relatively large volley, (*2*) followed by a smaller deflection (*3*). This tail has been found to be far more sensitive to an increase of stimulus frequency (Cf. LLOYD, 1941, for the same observation), which leads to a considerable decrease of its size, thus suggesting that it cannot have had the same origin as the first large volley. This view was strengthened by experiments in which the muscle was stretched by pulling on its tendon. To this increase of muscle tension the first, large volley responded with a considerable reduction in size whereas the effect of stretch on the small second potential was far less marked.

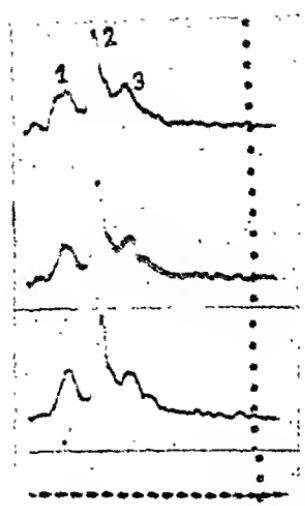


Fig. 27.

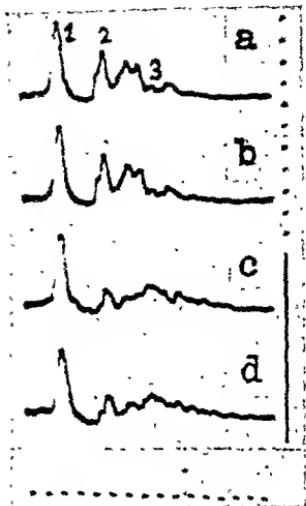


Fig. 28.

Backresponse from the muscles, recorded in dorsal root L 7 in response to stimulation of ventral L 7. 1, backresponse from proximal hip muscle. 2 and 3, backresponse from gastrocnemius, showing the spike potential (2), followed by an irregular tail (3).

Fig. 28. Discharge in dorsal root L 7 to stimulation of ventral L 7. 1, »Hering's phenomenon». 2 and 3, backresponse from gastrocnemius-soleus. a and b. Muscles unloaded. c. and d. Same during passive stretch of the muscles.

See text.

Fig. 28 illustrates an experiment in which, during stimulation of the ventral L 7, a strong pull was exerted on the tendo Achillis. In this case soleus was undenervated so that the second potential complex arises in the combined gastrocnemius-soleus muscle. It is preceded by a large volley (1) coming from the cut end of a proximal nerve to muscles above this region (the Hering phenomenon, HERING, 1882, LLOYD, 1942, GRANIT, LEKSELL and SKOGLUND, 1944, and others) and due to impulse transmission at this end. This discharge transmitted from the cut end (volley 1) may, of course, from the point of view of this experiment be regarded as an artefact and remains uninfluenced by what happens in the muscle. When, in records c and d, the pull is applied, the early part of the potential com-

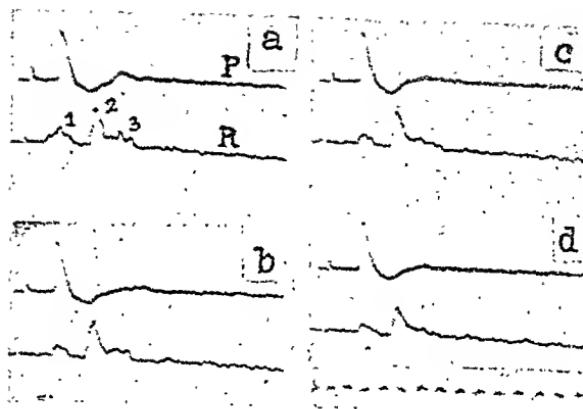


Fig. 29. Backresponse in the dorsal root L7 in response to stimulation of ventral L7. Simultaneous recording of the potential in the peroneal nerve.

a. Supramaximal stimulus strength. *P*, peroneal volley. *R*, backresponse, 1, from proximal hip muscle, 2 and 3, from gastrocnemius.

b—d. Successive reduction of stimulus strength.

plex diminishes whereas the tail of this potential is more resistant, indicating that the two components of the response are conducted in different fibres. In favour of this conclusion is also the somewhat higher threshold of the tail of the potential complex as compared with its initial phase.

This is demonstrated by the experiment in Fig. 29.

The backresponse has again been recorded from the dorsal root L7, the stimulating electrodes having been on the ventral L7. This is shown in record *a R*. In record *a P* the action potential in the peroneal nerve has been simultaneously picked up at a conduction distance which approximately corresponds to the distance from root to gastrocnemius muscle. This action potential has been led to the second beam of the cathode ray oscillograph. The alpha potential served as a control of threshold and stimulus strength and its conduction time could be directly compared with the latency of the backresponse..

In records 29 *a P* and *R* the stimulus strength is supramaximal. From *b* to *d* stimulus strength is successively decreased. The reduction of stimulus strength is accompanied by a slight-

ly greater reduction of the tail of the backresponse than of its first phase. This difference in threshold suggests that the tail of the backresponse possibly is initiated by activity in smaller efferent fibres than the early spike component.

In order to ascertain whether the efferent conduction actually takes place in the gamma fibres the different latent periods and conduction times must be considered.

The latent period of the large initial phase of the backresponse was in most experiments 3.5—4.0 msec. Within this time are included (i) conduction time from motor root to gastrocnemius, about 1.5 msec., (ii) approximately the same time back to the sensory root, and (iii) the delay in the muscle. The latent period of the tail part of the backresponse is approximately 1.5 msec. longer than the latency of the initial part. These additional 1.5 msec. may be explained by a slower rate of conduction in the efferent fibres.

Assuming the same conduction time in the afferent path and the same intramuscular delay for the spike and the tail of the backresponse, this difference in latency, however, is too small to correspond to efferent conduction in gamma fibres. If the impulses in the alpha fibres are conducted to the muscle in 1.5 msec., the gamma wave, having a propagation rate of approximately 40 per cent of alpha, will need 3.75 msec. and the latency differential will be 2.25 msec.

The tail of the backresponse cannot therefore be explained as due to the gamma fibres, without additional assumptions as to the time required for cross-stimulation inside the muscle and for afferent conduction. That the gamma fibres contribute to the late part of the backresponse is not excluded, however, and there are no obvious reasons why they, in this respect, should differ from other ventral root fibres to the muscles and not be able to cause any cross-stimulation with consequent backresponse.

It was attempted to obtain more information on these questions by using differential block of the efferent fibres. Since the backresponse is led off from the dorsal root, the block in this case had to be applied to the stimulated root itself, but it

was found that the roots were too short for being used for both stimulation and selective blocking.

As to the physiological significance of the backresponse, there is no evidence available. This question will not be considered in this connection. Attention has been directed to the properties of the backresponse chiefly because this phenomenon proved to be an unexpected complication in the attempts to isolate the gamma potential and study its rôle in muscle physiology.

b. The efferent gamma fibres and the afferent activity from the muscle proprioceptives.

The afferent impulses have in these experiments been recorded from the whole nerve. Isolation of single fibres was not attempted. Now it is clear that recording of massed impulses in this manner to some extent prevents evaluation of minor changes in the total activity. On the other hand, it is of some value to have the nerve undamaged and to obtain a general view of the total afferent activity. The polarization and pressure blocks as well as the threshold difference have been used to differentiate between alpha and gamma fibres.

The afferent discharge was recorded from the medial or lateral gastrocnemius nerves, which were carefully separated for 2—3 cm. from the main nerve stem in the popliteal fossa. It was noted that that part of the gastrocnemius muscle which is motorically innervated by either of these nerves also sends its afferents through the same nerve. Therefore the nerve was left unsevered and diphasic recording was used. This arrangement means that the whole sciatic stem can be blocked electrically, or by pressure, without interfering with the recording of the afferent activity from one of the branches separated *below* the block. Since the stimulating electrodes were on the ventral root the centrifugally directed volley suffered the full effect of the block used for differentiating between alpha and gamma fibres.

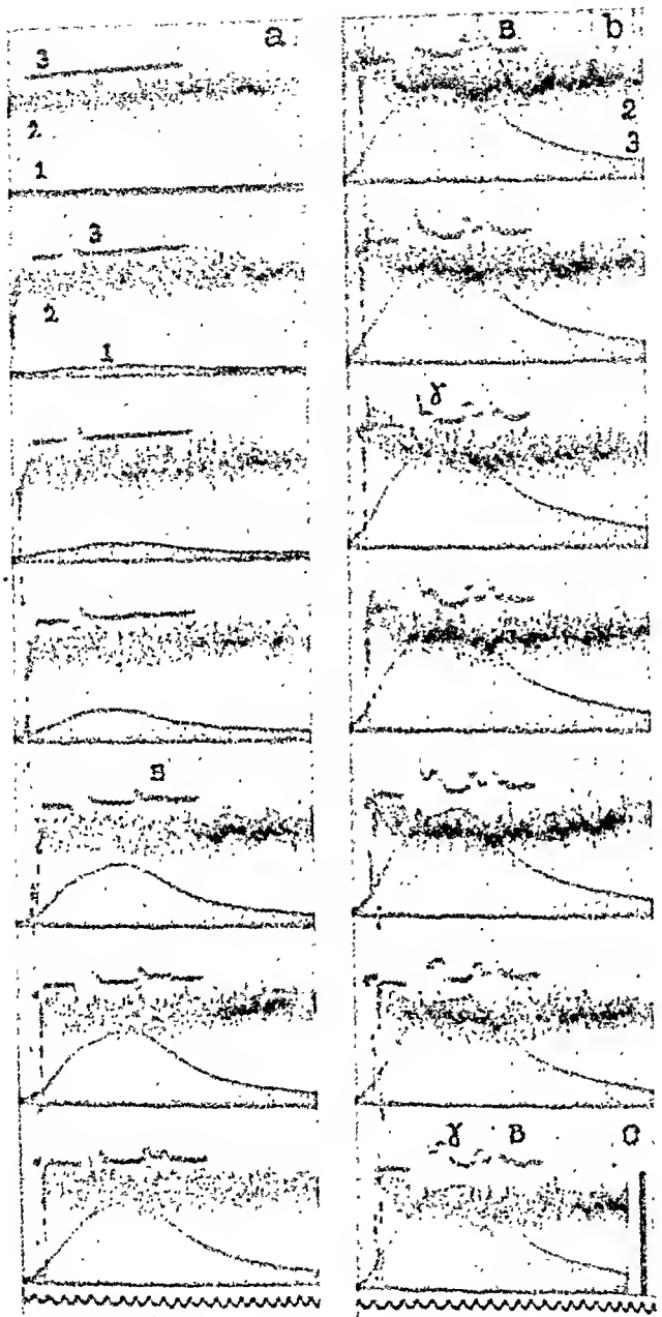


Fig. 30. Increase of afferent discharge from the muscle during contraction. Isometric twitch in gastrocnemius in response to stimulation of the sciatic nerve (a 1, b 3). Simultaneous recording of the afferent discharge in gastroc. med. (GM in diagram), (a 2, b 2).

When a muscle, kept under moderate initial tension, contracts isotonically the afferent discharge from the stretch receptors is reduced. During an isometric contraction, however, there is a considerable increase of the afferent response. A large number of experiments were carried out in order to ascertain to which extent, if at all, the high-threshold gamma fibres contributed to this increase. The stimulating electrodes were on the ventral root or on the sciatic nerve.

Fig. 30 illustrates an experiment with single twitches of the gastrocnemius and simultaneous records of the afferent activity in the medial gastrocnemius nerve in the manner already described. The sciatic nerve was stimulated. The muscle contraction and the discharge in the nerve from the muscle were reproduced transversally with the same sweep velocity (Fig. 30 *a*, 1 and 2). On the third beam (*a* 3) has been recorded the ventral root potential in L₇ with a faster sweep in order to possess one more control of stimulus strength and gamma activity. The polarity of the stimulus could be altered by means of a commutator. In this particular case, however, the difference between cathodal and anodal stimulation proved to be insignificant so that the cathode was all the time located to the distal electrode. The antidromically elicited gamma wave in the ventral root served as an approximate measure of the gamma contribution to the centrifugal volley.

In the uppermost picture of Fig. 30 *a* is reproduced the afferent activity (*2*) from the muscle in response to passive

The antidromic potential in the ventral root L₇ (v L₇) is also recorded, with rapid sweep (*a* 3).

Record *a*. Uppermost record before stimulation. Initial tension 250 gm. In the following records stimulus strength successively increased.

b. Contraction in the uppermost record just submaximal. In the following records successive increase of stimulus. *B*, backresponse from gastrocnemius.

c. Myograph calibration to 4 000 gm.

Time for contraction and afferent response in units of 5 msec.
Full description in text.

stretch before stimulation. The initial tension was about 250 gm. In the next record stimulation has started and a small alpha potential (β) is noticeable in the root. The muscle record (*1*) indicates a small contraction. This has sufficed to cause a considerable increase of the afferent activity in the nerve. The initial large potential, preceding the afferent discharge, consists of both the centrifugal stimulating volley and its back-response. The sweep speed is too low to separate them from each other. In the following records there is a successive increase of stimulus strength leading to an increased muscular tension and an increased afferent discharge: The alpha potential in the ventral root is seen to be succeeded by a small wave (*B*) which is the backresponse from the muscle. In the uppermost record of Fig. 30 *b*, the muscle twitch (*3*) is nearly maximal. The gamma potential has not yet appeared in the ventral root. Further increase of stimulus strength in the pictures leads to activation of the gamma wave, (γ). There is no significant further increase of the afferent discharge, the main increase having appeared already in Fig. 30 *a*, at an early stage in the experiment, long before the tension in the muscle was anywhere near its maximum value.

The same result was obtained with tetanic stimulation. After a slight initial decrease of the afferent activity followed the large increase which reached its maximum just before or simultaneously with the maximum of tension in the muscle. In a few cases a further increase of the afferent activity was observed upon further augmentation of stimulus strength but the effect was not regularly noted.

If the muscle is unloaded and slack there is also in some cases a small increase of afferent activity upon stimulation. In this case too the discharge begins before the tension is maximal and there is no definite augmentation of the effect when supramaximal stimuli are used.

An experiment of this kind is reproduced in Fig. 31. Single twitches were elicited in an unloaded muscle. Afferent activity and the alpha wave in the peroneal nerve were recorded. In Fig. 31 *a*, *1* shows the afferent discharge before the contraction.

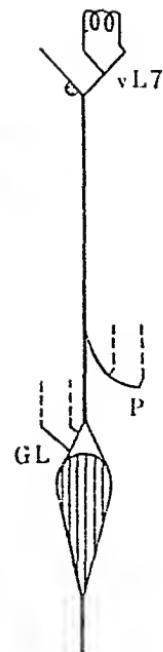
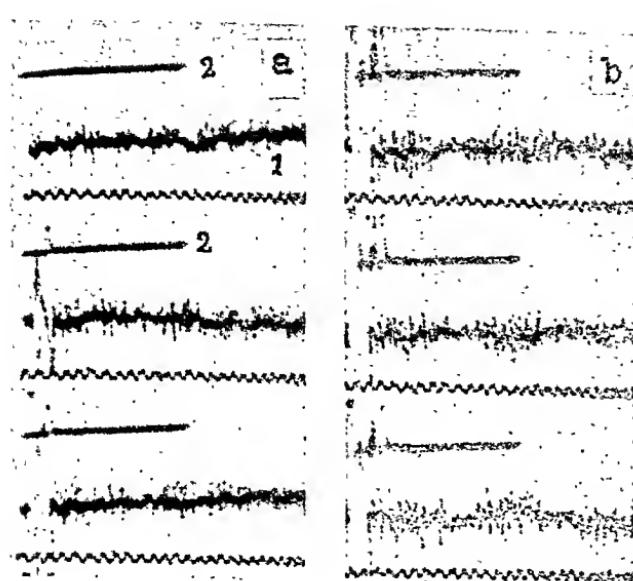


Fig. 31 *a* and *b*. Afferent activity from unloaded muscle. Simultaneous recording of the afferent activity from gastroc. lat. (GL in diagram) and of the α -potential in the peroneal nerve (P) in response to stimulation of the ventral root L 7 (v L 7). Uppermost record in *a* before stimulation. In the following records strength of stimulus successively increased. — Time marking for the afferent discharge (1) 5 msec.

The alpha potential in the peroneal nerve is recorded with a more rapid sweep (2). In the following records stimulus intensity has been successively increased and the alpha wave has reached its maximum value in the uppermost record in *b*. Further augmentation of stimulus strength does not markedly influence the afferent activity.

It is therefore clear that an effect of the gamma fibres on the afferent discharge from the muscle, if present, must be hidden under the large increase of afferent activity that sets in already in the early stages of a successively increasing tension in the muscle. This early effect cannot, of course, be due to the gamma fibres which are subthreshold for the stimuli used to elicit the weak contractions considered.

What is then the mechanism of origin of this massive afferent discharge? According to MATTHEWS contraction unloads the great majority of the receptors, both A 1 and A 2 endings, when the stimulus is submaximal. These receptors are lying in parallel with the muscle fibres. This should lead to a diminution of their activity. The B end organs, however, respond to stretch, independently of whether its origin is active or passive. Accordingly, the early increase of the afferent discharge in these experiments should be due to activation of the B endings. MATTHEWS (1933) and MORSON and PHILLIPS (1937) believe them to be localized, partly at least, to the tendon.

In order to find out whether the increase noted was due to end organs in the tendon the following experiment was carried out. The one nerve to the gastrocnemius was severed proximally, at the point where it joins the nerve stem. From this stump the afferent discharge was recorded and compared with the afferent activity in the second intact nerve. The ventral root L 7 was stimulated. Just as before, the initial discharge caused by passive stretch in the intact nerve, was greatly augmented upon stimulation with consequent increase of tension in the muscle. In the severed stump, however, which came from a region that had not received any centrifugally conducted impulses, the afferent activity diminished during stimulation of the muscle. Assuming end organs in the tendon to have caused the afferent discharge under discussion, it should not have made any difference whether the afferent discharge was recorded from the intact nerve or the stump. The fact that the afferent discharge only appeared in the intact nerve shows that the centrifugally conducted motor impulses were of some importance for the activity of the end organs and that these therefore must have been located to the muscle itself, not to its tendon. On the basis of MATTHEWS' concepts this means, either that B endings located to the muscle are responsible for the afferent discharge or that this submaximal increase of the afferent discharge is brought about by A 2 endings which need the efferent stimulus in order to discharge. The latter alternative implies that the intrafusal muscle fibres in

the muscle spindles are innervated by relatively large, low-threshold fibres belonging to the alpha group.

The experiments described have not given any definite support to the view that the gamma fibres influence the afferent discharge from the muscle proprioceptors. This negative result may, however, depend upon such effects having been concealed by the massive discharge just described or upon multiple innervation of the muscle spindles. AGDUHR (1916, 1919, 1939) and CUAJUNCO (1932) among others have maintained that there are several motor end organs located to the intrafusal muscle fibres.

If these are innervated both by coarse and small fibres, the experiments described may have been misleading. It is therefore important to try to isolate the gamma fibres by selectively blocking the alpha fibres. Such experiments, analogous with those already reported in the section on the muscle contraction, were therefore carried out in order to elucidate this point.

A motor root, L 7 or S 1, was stimulated supramaximally, and an electrical block or a pressure block applied to the sciatic nerve. The effect of the block was checked with the aid of peripheral records of the alpha wave and of ventral root records of the antidromically conducted wave which in addition reproduced the gamma potential. The latter procedure was only used with pressure blocks which affect conduction symmetrically in both directions.

An experiment of this type is reproduced in Fig. 32. The ventral root L 7 was stimulated and the afferent discharge picked up from the intact nerve to the medial head of gastrocnemius (Fig. 32 a 1). The muscle contraction was recorded on the third beam, without sweep, and again appears on the left in the film alongside the transversally recorded neurograms. The effect of the block was also controlled with a peroneal record of the alpha wave (2 P). This potential and the afferent discharge in the nerve from the muscle (1) were recorded on the double-beam cathode ray at the same sweep rates, and the sweep movement was started mechanically at intervals of about 1 or 2 sec.

In Fig. 32 *a* are shown two successive tetani, before application of the block. The uppermost beat of the sweep (*1*) shows the afferent activity before the contraction. It is elicited by the initial tension, caused by a load of 300 gm. Since there was no stimulation there is no alpha potential on sweep path 2. In the following records the afferent discharge is seen to undergo an increase during the contraction. The large initial deflections are a combination of efferent volley and backresponse. The peroneal alpha potential can be seen as a narrow spike, marked *P*. The maximal muscle tension during the two tetani corresponds to 2000 gm.

Fig. 32 *b* was taken 20 min. after application of the pressure block. The muscle contraction is now greatly reduced but can still be seen as a small protrusion of the shadow on the left. The peroneal alpha potential (*P*) is now about $\frac{1}{3}$ of its original size. The afferent activity, however, is still considerable during this small contraction. This suggests that it might be due to the active innervation of the sense organs rather than to the minute increase in tension in the muscle. The pressure block has not yet gone far enough to make it possible to decide whether this active innervation of the sense organs would be due to alpha or gamma fibres or to the combined action of both.

In the next set of records, Fig. 32 *c*, page 68, owing to the progress of the block, the potential (*P*) would hardly have been visible at all, had not the amplification been increased to 3 times the sensitivity used in the records *a* and *b*. The afferent activity, however, still increases considerably during the contraction as is evident by a comparison of record *c* 1, before the contraction, with record *c* 2. It is seen to follow in *c* 2 after the big initial wave, caused by efferent volley and back-response, and can still be called a vigorous discharge, of much the same order of magnitude as in Fig. 32 *a*. It is, of course, impossible to make quantitative comparisons between massed discharges of this character. In Fig. 32 *d*, taken shortly after record *c*, a similar increase of the different response during contraction may be seen.

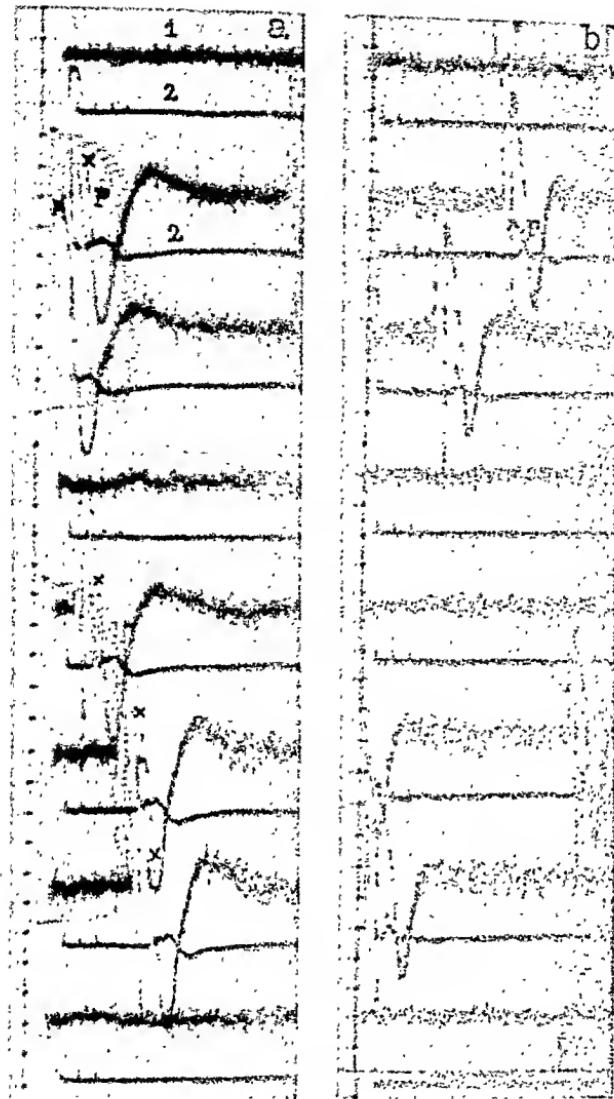


Fig. 32 *a* and *b*. Afferent discharge from the muscle and differential block of the α -fibres. Isometric tetanus in gastrocnemius to supramaximal stimulation of the ventral root L 7 (v L 7 in diagram). Simultaneous recording of the afferent activity (*a* 1) at gastroc. med. (GM in diagram) and the α -potential in the peroneal nerve (P in diagram).

a. Two successive tetani before application of the pressure block on the sciatic. Stimulus frequency approximately 23 per sec. 1, the afferent activity in gastroc. med. before stimulation. Passive tension 300 gm. P, α -potential in peroneal nerve, recorded on the second beam of the cathode ray tube (2); X, crest of α -potential.

b. Same as *a* after a blocking time of approximately 20 min.

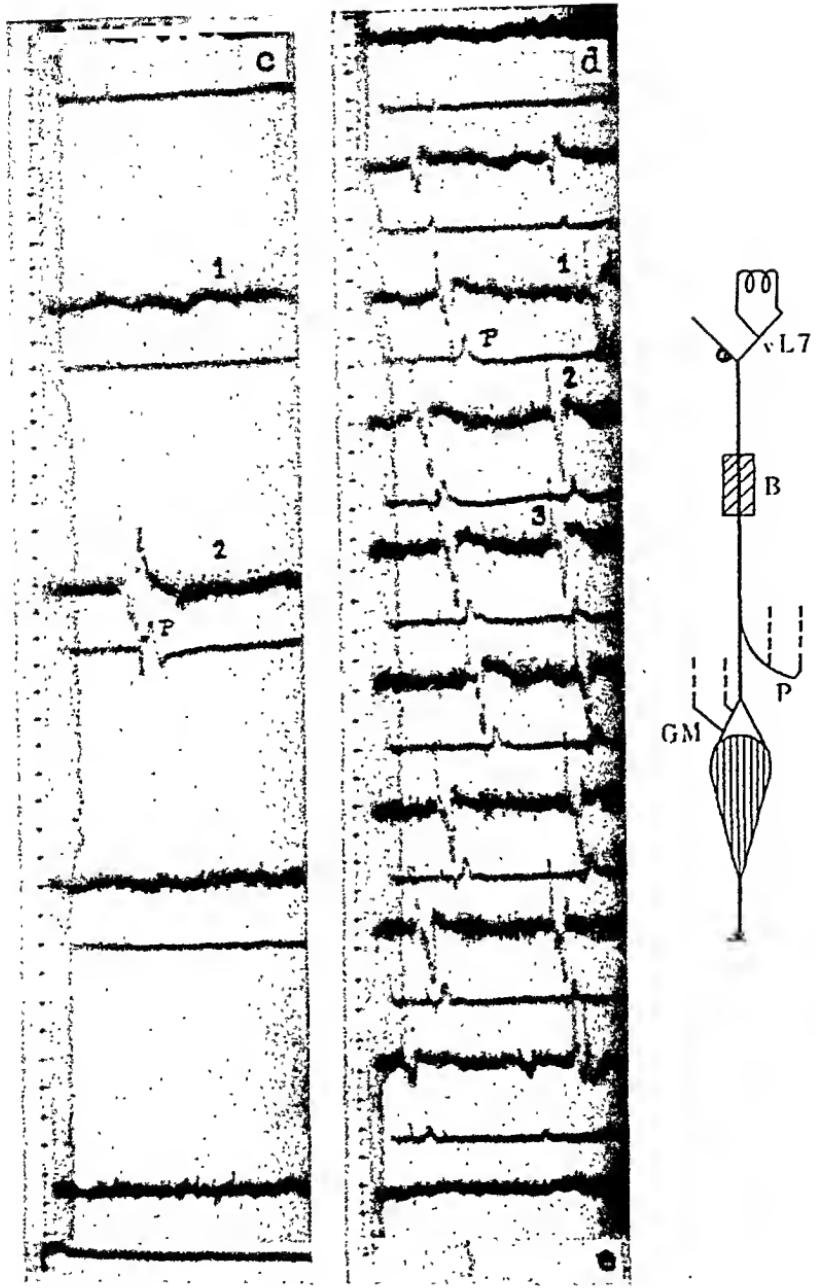


Fig. 32 c and d.

c. Isometric tetanus after a blocking time of approximately 30 min. Maximal sensitivity of the myograph. Amplification of the peroneal volley (**P**) 3 times higher than in fig. 32 *a* and *b*. Frequency of the stimuli the same as in 32 *a* and *b*, 23 per sec.

Thus, although the block has eliminated most of the contraction the afferent activity is increased during the contractile phase, much as if the descending volley had stimulated the afferent end organs. It is easily shown that the impulses really constitute an afferent discharge from the muscle. One way is to remove the load from the muscle. Then the impulses disappear. They can only originate in a muscle subjected to some initial tension. This property would place the end organs or afferents concerned among the types which MATTHEWS has called A 2 endings.

Similar results were obtained with the polarization block. Despite practically complete removal of the contraction, there was a considerable increase of the afferent discharge during stimulation of the efferents. Some difficulties are often encountered with this type of block owing to the repetitive discharges from the blocked region. There was first a maximal tetanus in a certain phase of polarization. This, however, disappeared upon further strengthening of the block. But repetitive activity in the sensory fibres remained for a much longer time (Cf. SKOGLUND, 1942) and interfered with the afferent effects caused by stimulation of the efferent root, thereby making it difficult to separate the former from the latter. But it was found possible to evade these difficulties and differentiate the afferent discharge from the impulses from the block by diminishing the load on the muscle or cutting the motor nerve distal to the leads. With the aid of these controls it was shown that stimulation of the efferent nerve to the muscle caused a vigorous afferent discharge at a stage when no muscle contraction could be recorded. Most experiments, however, were carried out with the less complicated pressure block.

- d. Tetanus after approximately 35 min. Downwards gradually increasing stimulus strength (1, 2, 3). Stimulus frequency approximately 45 per sec.
- e. Myograph calibration 50 gm.

Full description in text.

An experiment with pressure block on the sciatic and single twitches instead of tetanic stimulation is shown in Fig. 33. The records refer to the last phase of the experiment. The ventral root S 1 was stimulated. The afferent activity from the muscle (Fig. 33, *a*, 2, 4 and *b*, 2, 4) was recorded in the same way as before. The pressure was moderate so that the effect developed very gradually. The records were taken respectively 60 and 75 min. after application of the block. As a check on the block an antidromic ventral root potential in L 7 was alternately recorded.

In Fig. 33 *a* 1 is shown the antidromic potential in the root. There is a great reduction of the alpha wave, seen as a narrow spike of low amplitude. The gamma wave (γ) is clearly visible. The afferent discharge, corresponding to this stage, is shown just below this record and marked 2. Since the stimulus has been antidromic (from the cut peroneal), there is no muscle contraction, and the afferent discharge thus represents the effect of passive stretch amounting to a tension of about 200 gm. The initial wave is merely a shock artefact. The stimulus was then switched over to the root. Because of the block the muscle contraction *M* had diminished to less than 20 gm from an original value of 1750 gm, or thus been reduced to about 1 per cent. By comparing *a* 2 with *a* 4 it can be seen that the afferent discharge increases during stimulation, despite the smallness of the contractile effect.

In the records *b* further development of the block has led to complete disappearance of the antidromic volley (*b* 1), despite maximal amplification. But efferent stimulation of the muscle still led to a well marked afferent discharge, as may be seen by comparing records *b* 2 and *b* 4.

On the basis of these experiments it seems safe to conclude that at a stage when the block has succeeded in removing practically all the fibres, responsible for the muscle contraction, a centrifugal volley through the thin gamma fibres still causes a considerable increase in the afferent discharge from the muscle, provided that the muscle is subjected to a certain amount of passive stretch.

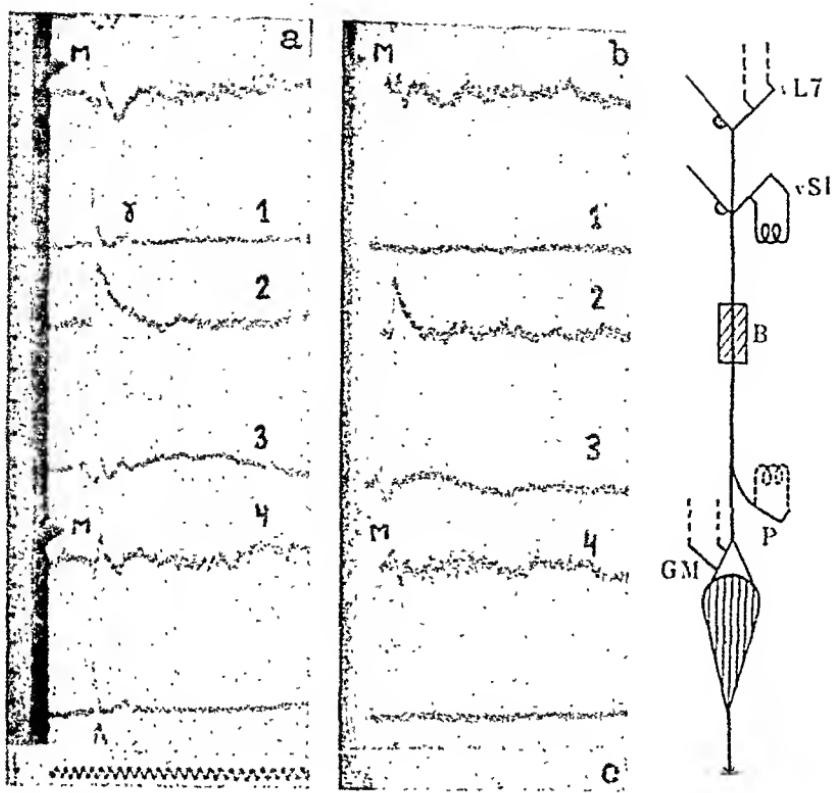


Fig. 33. Afferent discharge after differential blocking of the alpha fibres. Isometric twitch in gastrocnemius in response to stimulation of the ventral root S 1. (v S 1) Compression block (B) on the sciatic nerve. Block controlled by antidromic volleys from the peroneal nerve to v L 7. Twitch before blocking 1 750 gm.

a. After a blocking time of 60 min. 1, antidromic volley recorded at v L 7. 2, afferent discharge in gastroc. med. to passive tension (200 gm.), preceded by large shock artefact. *M*, muscle twitch after switching over of stimulus to v S 1. Tension approximately 17 gm. 3, record from v L 7 to stimulation of v S 1, (shock artefact). 4, afferent discharge from the muscle during contraction *M*.

b. After a blocking time of 75 min. 1, record from v L 7 to stimulation of peroneal nerve. No potential visible. 2, afferent discharge from the muscle during passive stretch. *M*, muscle twitch to stimulation of v S 1. 3, record from v L 7 to stimulation of v S 1. 4, afferent discharge during twitch.

c. Calibration of myograph to 20 gm.

See text.

In still another way it has been possible to substantiate this conclusion: if stimuli of successively increasing strengths are applied to the root it is found that the afferent discharge does not appear before the threshold for the gamma fibres is reached.

This can be seen by turning back to Fig. 32 *d*, p. 68. The small tetanic contraction, left in the last stage of the block, was elicited with stimuli of gradually increasing strength. In the sweep movement, marked 1, there is only a very slight increase of the afferent discharge. The muscle contraction, however, is nearly maximal, and so is the small remaining alpha volley in the peroneal nerve (*P*). The next beat of the sweep circuit brings out the augmentation of the afferent discharge (2), and it still increases upon further increase of stimulus strength in 3. But these afferent effects are only accompanied by a very small addition to contractile tension, something of the order of a few grams, despite the fact that the majority of gamma fibres gradually entered the field of activity. It is therefore clear that the increase of the afferent discharge was due to the stimuli gradually becoming suprathreshold for the gamma fibres, and that their effect upon the muscular tension was practically *nil*.

DISCUSSION

In the first section it was demonstrated that a component potential corresponding to the group of small ventral root fibres to the skeletal muscles in cats (ECCLES and SHERRINGTON, 1930) could be recorded by leading off from the roots. The reasons why such attempts (O'LEARY, HEINBECKER and BISHOP, 1934) previously have been unsuccessful may have been that the conduction distances were too short.

The small potential wave was called the »efferent gamma» wave in agreement with the nomenclature of ERLANGER and GASSER (1924). In more recent work by GASSER and his collaborators (GASSER, 1937, GRUNDFEST and GASSER, 1938, GRUNDFEST, 1939) other criteria than conduction velocity, such as, for instance spike duration and afterpotentials, have been used for classification of the A, B and C groups of fibres, but these have not been included in the analysis of this new wave in the efferent output. It has been referred to the gamma elevation of the A group on account of its conduction velocity which is about 40 per cent of that of the alpha wave. This need not mean that the motor gamma fibres in all respects could be compared with the sensory gamma fibres. Recently important differences between sensory and motor fibres have been noted, for instance with regard to accommodation and repetitiveness (ERLANGER and BLAIR, 1938, in frogs, SKOGLUND, 1942, in cats, KUGELBERG, 1944, in man) or with regard to pressure (DENNY-BROWN and BRENNER, 1944). For the time being such factors will be left out of consideration but they should not be left out of sight.

Section II was devoted to an analysis of the effects caused by gamma innervation. We have seen that the results could be briefly summarized in the statement that highly selective activation of the efferent gamma fibres, so far from causing a definite motor

effect, on the contrary, gave rise to an *afferent* discharge from the muscle, provided that the latter was subjected to a certain amount of passive stretch. In slack muscles no effect whatever could be ascribed to these fibres. Whether the gamma fibres are unique among the efferent fibres in causing an afferent response cannot be stated. Some of the experiments described above (p. 61, 62) indicate that larger fibres in the motor range may have a similar function.

In the case of the gamma fibres this influence on the afferent activity of the muscle appears to be their exclusive task. They are evidently connected to the sensory apparatus as a regulating mechanism. This fact is in itself of considerable general interest and recalls the organisation of the retina and the organ of Corti to both of which centrifugal fibres are said to pass (RAMON Y CAJAL, 1909, 1911). Nothing is known concerning their function. The centrifugal gamma fibres in the ventral roots must somehow sensitize or directly activate structures which discharge under the conditions demonstrated by MATTHEWS to hold for his so-called A 2 end organs believed to be the annulo-spiral endings of the muscle spindles. They respond during active isometric contraction of a tense muscle and remain silent when the muscle is slack. The gamma fibres may well be motor for the intrafusal muscle fibres.

It is difficult to explain the mode of action of the gamma fibres in detail. But two main alternatives deserve to be briefly mentioned. A sensory end organ, according to BERNHARD, GRANIT and SKOGLUND (1942), may be schematically described as consisting of two parts, a primary mechanism responding to the adequate stimulus and a secondary mechanism initiating the discharge in the nerve. The latter they hold to be an electrical generator potential conducted electrotonically down the afferent nerve (BERNHARD 1942).

In this particular case the gamma fibres may facilitate the discharge from the afferents either by attacking the primary mechanism or by contributing to the generator potential. In the former case it would have to be assumed that the impulses in the gamma fibres cause a minute increase of tension in the intrafusal

muscle fibres thereby sensitizing the receptor so that its threshold to stimulation by stretch is lowered. In the latter case the efferent potentials would act directly by depolarizing some critical region of the receptive path. There is nothing inherently improbable in the latter view. It is only necessary to recall the mechanism of the backresponse in order to understand how gamma fibres could stimulate directly by way of a potential field in the intrafusal fibres or in other elements of the muscle. The dependence of the response upon the state of tension of the muscle might then be explained by the different amount of steady potential which muscles are known to set up when passively stretched (EINTHOVEN and RADEMAKER, 1916, BJURSTEDT and SCHMITERLÖW, 1943). At the moment it is impossible to go beyond these general suggestions as to the mechanism of the excitatory effect of the gamma fibres.

Selective stimulation of the gamma fibres, by utilizing their high threshold and by removing alpha impulses with the aid of a pressure or polarization block, has been used above in order to throw light upon the effects of gamma innervation on the muscle. As to the effect of the afferent response, elicited by the gamma fibres, on the reflex activity of the spinal cord nothing is known. It remains for future work to elucidate the part played by the gamma fibres in reflex posture and reflex movement.

The experiments described in this work do not support the view of HÄGGQVIST (1938, 1939, 1940) that the group of small ventral root fibres maintain a »tonic» contraction by direct stimulation of muscle fibres. It has not been possible to demonstrate any definite contractile effect of the gamma fibres, despite the fact that motor alpha activity was removed by the methods described. In as much as reflex »tonus», as is well known since the work of SHERRINGTON (1915) and LIDDELL and SHERRINGTON (1924, 1925), is principally based on the myotatic reflex, it is also interesting to note that the phasic component of this reflex recently has been shown to pass through the two-neuron arc of RENSHAW (1940) and to be conducted in fast and large fibres (LLOYD, 1943). On the other hand, the fact that the gamma fibres are regulators of the afferent discharge

suggests that they are of importance for the postural reflexes, maintaining »tonus«. If this effect is excitatory or inhibitory we do not know.

Finally a few words might be added about the alleged sensory function by direct centripetal conduction of fibres in the motor roots (SHAW, 1924, FOERSTER, 1927, LEHMANN, 1936, and others). The proofs are not convincing. The evidence is largely circumstantial and rests chiefly on the fact that pain sensations may remain after severance of the dorsal roots. In some cases the explanation may be that some strands of the dorsal root filaments still were intact. There are also observations by a number of authors (MEYER, 1921, 1926, FRÖLICH and MEYER, 1922, DAVIS and POLLOCK, 1930) which are contradictory to the view that the ventral root fibres hold true afferents. FOERSTER's (1927) observation that electrical stimulation of the thoracic ventral roots evokes painful sensations may be explained by spread of current or by SHERRINGTON's (1894) old observation that recurrent fibres from the dorsal roots sometimes are found in the adjacent ventral roots. The possible occurrence of such recurrent fibres from the dorsal root ganglia (FOERSTER and GAGEL, 1933) is without consequence for the present problem.

In a few cases of posterior root sections for painful osteo-arthritis of the hip I have, during operation, stimulated anterior lumbar roots with induction shocks, after application of a novocaine block below the stimulated region. No sensations were experienced by these patients, when stimuli were used of up to 20 times the value necessary for a threshold muscular contraction, tested before application of the block.

There is thus no valid reason to ascribe any direct afferent function to the gamma fibres. The afferent effects caused by the small ventral root fibres to skeletal muscle are indirect and due to their facilitatory influence on the proprioceptive sensory mechanism.

SUMMARY

This work describes an electrophysiological analysis of the group of small ventral root fibres to skeletal muscle, carried out with cathode ray oscillograph, amplifier and a special torsion wire myograph operated on electrical principles. Chiefly cats, but also some frogs were used.

The study is divided into two sections. Section I is devoted to an attempt to reproduce the wave of action that must correspond to the passage of a volley of impulses through the small fibres but which hitherto has not been found. Section II deals with the effect of excitation of the small fibres.

Section I.

It was found possible to record the action potential of the small fibres in the ventral roots, sometimes also, though far less regularly, in a peripheral portion of the sciatic nerve.

Relative to the conduction velocity of the coarse alpha fibres, taken as 100 m. p. s., the group of small fibres was found to have a conduction velocity of approximately 38 m. p. s., for the fastest fibres, down to approximately 20 m. p. s. for the slowest fibres. On the basis of this relative conduction rate the action potential of the small fibres was called the efferent *gamma* wave.

The stimulus threshold of the largest gamma fibres was found to be approximately 3.9 times that of the largest alpha fibres.

A correlation between electrophysiological and histological data was established.

It was further found possible to obtain selective gamma fibre activity by blocking the alpha fibres with the aid of pressure or polarization blocks, the action potential being used as an index of the degree of selectivity of the block.

Section II.

Selective activation of the gamma fibres was used in this section for the study of the effects of gamma innervation.

With fairly selective blocking of the alpha fibres and activation of the gamma group the isometric contraction was found reduced to some 1 or 2 p. c. (10—20 gm) of its maximum value. Since this small contractile effect may have been caused by a few remaining alpha fibres, it is concluded that no definite motor function can be demonstrated for the gamma fibres.

The gamma fibres, however, had a very definite effect on the *afferent* discharge from the muscle proprioceptors. Selective activation of the gamma fibres led to a considerable increase of the afferent discharge, provided that the muscle was subjected to a certain amount of stretch. In slack muscles this effect did not appear.

It is concluded that the gamma fibres serve as regulators of sensory activity originating in the muscle, and the mechanism of this effect is discussed.

REFERENCES

AGDUHR, E. Morphologischer Beweis der doppelten (plurisegmentalen) motorischen Innervation der einzelnen quergestreiften Muskelfasern bei den Säugetieren. *Anat. Anz.*, 1916, 49: 1—13.

AGDUHR, E. Über die plurisegmentelle Innervation der einzelnen quergestreiften Muskelfasern. *Anat. Anz.*, 1919, 52: 273—291.

AGDUHR, E. On the innervation of cross-striated muscle-fibres. *Upsala Läk.-förcn. förhandl.*, 1939, 45: 399—418.

ARNELL, N. Untersuchung über die Dicke des Achsenzyinders und der Markscheide in nicht fixierten Spinalnerven des Menschen und des Hundes sowie über den Einfluss von Formalinfixierung, Paraffineinbettung und Ag-Imprägnierung auf dieselbe. *Acta psychiat. et neurol.*, 1936, 11: 5—25.

BENTLEY, F. H., and SCHLAPP, W. Experiments on the blood supply of nerves. *J. Physiol.*, 1943, 102: 62—71.

BENTLEY, F. H., and SCHLAPP, W. The effects of pressure on conduction in peripheral nerve. *J. Physiol.*, 1943, 102: 72—82.

BERNHARD, C. G. Isolation of retinal and optic ganglion response in the eye of *Dytiscus*. *J. Neurophysiol.*, 1942, 5: 32—48.

BERNHARD, C. G., GRANIT, R., and SKOGLUND, C. R. The breakdown of accommodation — nerve as model sense-organ. *J. Neurophysiol.*, 1942, 5: 55—68.

BISHOP, G. H., and GILSON, A. S. Action potentials accompanying the contractile process in skeletal muscle. *Am. J. Physiol.*, 1927, 82: 478—495.

BISHOP, G. H., and GILSON, A. S. Action potentials from skeletal muscle. *Am. J. Physiol.*, 1929, 89: 135—151.

BISHOP, G. H., and HEINBECKER, P. Differentiation of axon types in visceral nerves by means of the potential record. *Am. J. Physiol.*, 1930, 94: 170—200.

BISHOP, G. H., and HEINBECKER, P. The afferent functions of non-myelinated or C fibers. *Am. J. Physiol.*, 1935, 114: 179—193.

BISHOP, G. H., HEINBECKER, P., and O'LEARY, J. The function of the non-myelinated fibers of the dorsal roots. *Am. J. Physiol.*, 1933, 106: 647—669.

BISHOP, G. H., and O'LEARY, J. B and C nerve fibers. *Am. J. Physiol.*, 1939, 126: 434.

BJURSTEDT, H., and SCHMITERLÖW, C. G. Stretch potentials of skeletal muscle. *Acta physiol. Scandinav.*, 1943, 6: 359—367.

BJÖRKMAN, A., and WOHLFART, G. Faseranalyse der Nn. oculomotorius, trochlearis und abducens des Menschen und des N. abducens verschiedener Tiere. *Ztschr. mikr.-anat. Forsch.*, 1936, 39: 631—647.

CLARK, D., HUGHES, J., and GASSER, H. S. Afferent function in the group of nerve fibers of slowest conduction velocity. *Am. J. Physiol.*, 1935, 114: 69—76.

CUAJUNCO, F. The plurisegmental innervation of neuromuscular spindles. *J. comp. Neurol.*, 1932, 54: 205—226.

CUSHING, H. Some experiments and clinical observations concerning states of increased intracranial tension. *Am. J. M. Sc.*, 1902, 124: 375—400.

DAVIS, L., and POLLOCK, L. J. The peripheral pathway for painful sensation. *Arch. Neurol. Psychiat.*, 1930, 24: 888—898.

DENNY-BROWN, D., and BRENNER, C. Lesion in peripheral nerve resulting from compression by spring clip. *Arch. Neurol. Psychiat.*, 1944, 52: 1—19.

DUN, F. T., and FENG, T. P. Studies on the neuromuscular junction. XX. The site of origin of the junctional after-discharge in muscles treated with guanidine, barium or eserine. *Chinese J. Physiol.*, 1940, 15: 433—444. Cited by LLOYD 1942.

DOGIEL, A. S. Die Endigungen der sensiblen Nerven in den Augenmuskeln und deren Sehnen beim Menschen und den Säugetieren. *Arch. mikr. Anat.*, 1906, 68: 501—524. Cited by O'LEARY *et al.* 1934.

ECCLES, J. C., and SHERRINGTON, C. S. Numbers and contraction-values of individual motor-units examined in some muscles of the limb. *Proc. roy. Soc.*, 1930, 106 B: 326—357.

EINTHOVEN, W., and RADEMAKER, A. C. A. Über die angebliche positive Stromschwankung in der Schildkrötenvorkammer bei Vagusreizung nebst Bemerkungen über den Zusammenhang zwischen Kontraktion und Aktionsstrom. *Pflüger's Arch. ges. Physiol.*, 1916, 166: 109—143.

ERLANGER, J. The interpretation of the action potential in cutaneous and muscle nerve. *Am. J. Physiol.*, 1927, 82: 644—655.

ERLANGER, J., in Erlanger, J., and Gasser, H. S. *Electrical signs of nervous activity*. Philadelphia, Univ. Penn. Press, 1937, 221 pp.

ERLANGER, J., BISHOP, G. H., and GASSER, H. S. Experimental analysis of the simple action potential wave in nerve by the cathode ray oscillograph. *Am. J. Physiol.*, 1926, 78: 537—573.

ERLANGER, J., BISHOP, G. H., and GASSER, H. S. The action potential waves transmitted between the sciatic nerve and its spinal roots. *Am. J. Physiol.*, 1926, 78: 574—591.

ERLANGER, J., and BLAIR, E. A. Comparative observations on motor and sensory fibers with special reference to repetitiousness. *Am. J. Physiol.*, 1938, 121: 431—453.

ERLANGER, J., and GASSER, H. S. The compound nature of the action current of nerve as disclosed by the cathode ray oscillograph. *Am. J. Physiol.*, 1924, 70: 624—666.

ERLANGER, J., and GASSER, H. S. The action potential in fibers of slow conduction in spinal roots and somatic nerves. *Am. J. Physiol.*, 1930, 92: 43—82.

FOERSTER, O. *Die Leitungsbahnen des Schmerzgefühls und die chirurgische Behandlung der Schmerzzustände*. Berlin, Urban & Schwarzenberg, 1927, 360 pp.

FOERSTER, O., and GAGEL, O. Über affrente Nervenfasern in den vorderen Wurzeln. *Ztschr. ges. Neurol. Psychiat.*, 1933, 144: 313—324.

FRÖLICH, A., and MEYER, H. H. Zur Frage der visceralen Sensibilität. *Ztschr. ges. exper. Med.*, 1922, 29: 87—113.

GASKELL, W. H. On the structure, distribution and function of the nerves which innervate the visceral and vascular systems. *J. Physiol.*, 1886, 7: 1—80.

GASSER, H. S. The relation of the shape of the action potential of nerve to conduction velocity. *Am. J. Physiol.*, 1928, 85: 699—711.

GASSER, H. S. Conduction in nerves in relation to fiber types. *A. Research nerv. ment. Dis., Proc.*, 1935, 15: 35—59.

GASSER, H. S., in Erlanger, J., and Gasser, H. S. *Electrical signs of nervous activity*. Philadelphia, Univ. Penn. Press, 1937, 221 pp.

GASSER, H. S., and ERLANGER, J. The rôle played by the constituent fibers of a nerve trunk in determining the form of its action potential wave. *Am. J. Physiol.*, 1927, 80: 522—547.

GASSER, H. S., and ERLANGER, J. The rôle of fiber size in the establishment of a nerve block by pressure or cocaine. *Am. J. Physiol.*, 1929, 88: 581—591.

GASSER, H. S., and GRUNDFEST, H. Axon diameters in relation to the spike dimensions and the conduction velocity in mammalian A fibers. *Am. J. Physiol.*, 1939, 127: 393—414.

GRANIT, R., LEKSELL, L., and SKOGLUND, C. R. Fibre interaction in injured or compressed region of nerve. *Brain*, 1944, 67: 125—140.

GRUNDFEST, H. The properties of mammalian B fibers. *Am. J. Physiol.*, 1939, 127: 252—262.

GRUNDFEST, H., and GASSER, H. S. Properties of mammalian nerve fibers of slowest conduction. *Am. J. Physiol.*, 1938, 123: 307—318.

GÖPFERT, H., and SCHAEFER, H. Die mechanische Latenz des Warmblütermuskels nebst Beobachtungen über die Muskelzuckung und den Aktionsstrom. *Pflüger's Arch. ges. Physiol.*, 1941, 245: 60—71.

VAN HARREVELD, A., and MARMONT, G. The course of recovery of the spinal cord from asphyxia. *J. Neurophysiol.*, 1939, 2: 101—111.

HEINBECKER, P., O'LEARY, J., and BISHOP, G. H. Nature and source of fibers contributing to the saphenous nerve of the cat. *Am. J. Physiol.*, 1933, 104: 23—35.

HERING, E. Beiträge zur allgemeinen Nerven und Muskel-Physiologie. IX. Ueber Nervenreizung durch den Nervenstrom. *Sitzungsber. d. k. Akad. d. Wissensch. Math.-naturw. Cl.*, 1882, 85: 237—275.

HINSEY, J. C. The innervation of skeletal muscle. *Physiol. Rev.*, 1934, 14: 514—585.

HURSH, J. B. Conduction velocity and diameter of nerve fibers. *Am. J. Physiol.*, 1939, 127: 131—139.

HÄGGQVIST, G. Faseranalyse der vorderen Spinalwurzeln des Macacus rhesus. *Ztschr. mikr.-anat. Forsch.*, 1937, 42: 33—69.

HÄGGQVIST, G. Über die Frage der Existenz eines dritten Systems efferenter Fasern des Zentralnervensystems. *Verhandl. d. Anat. Gesellsch.*, 24—27 Aug. 1937, *Ergänz. Heft. Anat. Anz.*, 1938, 85: 191—196.

HÄGGQVIST, G. Zur Kenntnis einer doppelten cerebrospinalen Innervation der Skelettmuskeln. *Ztschr. mikr.-anat. Forsch.*, 1938, 43: 491—508.

HÄGGQVIST, G. Skelettmuskernas toniska innervation. *Hygiea*, 1938, 100: 224—247.

HÄGGQVIST, G. The nervous substratum of muscle tone. *Acta orthop. Scandinav.*, 1939, 10: 255—277.

HÄGGQVIST, G. A contribution to the question of the nervous and muscular substratum of the muscle tone. *Acta med. Scandinav.*, 1940, 104: 8—20.

KATZ, B. and KUFFLER, S. W. Multiple motor innervation of the frog's sartorius muscle. *J. Neurophysiol.*, 1941, 4: 209—223.

KUGELBERG, E. Accommodation in human nerves and its significance for the symptoms in circulatory disturbances and tetany. *Acta physiol. Scandinav.*, 1944, Suppl. 24: 105 pp.

LANGLEY, J. N. The nerve fibre constitution of peripheral nerves and of nerve roots. *J. Physiol.*, 1922, 56: 382—395.

LEHMANN, W. Chirurgische Therapie bei Erkrankungen und Verletzungen des Nervensystems. In Bumke, O., and Foerster, O. *Handb. d. Neurologie*, 1936, 8: 90—265.

LIDDELL, E. G. T., and SHERRINGTON, C. S. Reflexes in response to stretch (myotatic reflexes). *Proc. roy. Soc.*, 1924, 96 B: 212—242.

LIDDELL, E. G. T., and SHERRINGTON, C. S. Further observations on myotatic reflexes. *Proc. roy. Soc.*, 1925, 97 B: 267—283.

LLOYD, D. P. C. Centripetal discharges in dorsal and ventral roots following stimulation of muscle by ventral root volleys. *Proc. Soc. exp. Biol. N. Y.*, 1941, 47: 44—47.

LLOYD, D. P. C. Stimulation of peripheral nerve terminations by active muscle. *J. Neurophysiol.*, 1942, 5: 153—165.

LLOYD, D. P. C. Conduction and synaptic transmission of the reflex response to stretch in spinal cats. *J. Neurophysiol.* 1943, 6: 317—326.

MASLAND, R. L., and WIGTON, R. S. Nerve activity accompanying fasciculation produced by prostigmin. *J. Neurophysiol.*, 1940, 3: 269—275.

MATTHEWS, B. H. C. The response of a single end organ. *J. Physiol.*, 1931, 71: 64—110.

MATTHEWS, B. H. C. The response of a muscle spindle during active contraction of a muscle. *J. Physiol.*, 1931, 72: 153—174.

MATTHEWS, B. H. C. Nerve endings in mammalian muscle. *J. Physiol.*, 1933, 78: 1—53.

MEYER, A. W. Verlaufen sensible Fasern in den vorderen Wurzeln? *Zentralbl. Chir.*, 1921, 49: 1790—1793.

MEYER, A. W. Über die fraglichen sensiblen Fasern der vorderen Wurzeln. *Deutsche Ztschr. Chir.*, 1926, 199: 38—65.

MORSON, S., and PHILLIPS, G. Contralateral effects from tension receptors in skeletal muscle. *J. Physiol.*, 1937, 88: 199—212.

O'LEARY, J., HEINBECKER, P., and BISHOP, G. H. Analysis of function of a nerve to muscle. *Am. J. Physiol.*, 1934, 110: 636—658.

RAMÓN Y CAJAL, S. *Histologie du système nerveux de l'homme et les vertébrés*. Paris, Maloine, 1909, 1911; 2 vols.

RENSHAW, B. Activity in the simplest spinal reflex pathways. *J. Neurophysiol.*, 1940, 3: 373—387.

REXED, B. Contributions to the knowledge of the postnatal development of the peripheral nervous system in man. *Acta Psychiat. et neurol.*, 1944. Suppl. 33: 206 pp.

ROSENBLUETH, A., WILLS, J. H., and HOAGLAND, H. The slow components of the electrogram of striated muscle. *Am. J. Physiol.*, 1941, 133: 724—735.

SCHAEFER, H., and SCHMITZ, W. Zur Physiologie des Nervenschwirrens und der Kitzclämpfindung. *Ztschr. Sinnesphysiol.*, 1933, 64: 161—174.

SHAW, R. C. A study of intractable pain relative to rhizotomy and spinal section. *Brit. J. Surg.*, 1924, 11: 648—675.

SHERRINGTON, C. S. On the anatomical constitution of nerves to skeletal muscles, with remarks on recurrent fibers in the ventral spinal nerve-root. *J. Physiol.*, 1894, 17: 211—258.

SHERRINGTON, C. S. Postural activity of muscle and nerve. *Brain*, 1915, 38: 191—234.

SKOGLUND, C. R. The response to linearly increasing currents in mammalian motor and sensory nerves. *Acta Physiol. Scandinav.*, 1942, Suppl. 12: 75 pp.

SWENSSON, A. Über die Kaliberverhältnisse in den vorderen Rückenmarkswurzeln beim Menschen. *Ztschr. mikr.-anat. Forsch.*, 1938, 44: 187—206.

WILLS, J. H. Speed of responses of various muscles of cats. *Am. J. Physiol.*, 1942, 136: 623—628.

ACTA PHYSIOLOGICA SCANDINAVICA

VOL. 10. SUPPLEMENTUM XXXII

*From the Chemical Institution, Karolinska
Institutet, Stockholm*

A DIELECTRIC
STUDY OF
POLYNUCLEOTIDES

By

GUNNAR JUNGNER

STOCKHOLM 1945



Contents

	Page
Introduction	5
 <i>Part I. Theory.</i>	
Chapter I. Dielectric polarization	11
Chapter II. Computation of dipole moment and time of relaxation	17
 <i>Part II. Method.</i>	
Chapter III. The possibilities of determining anomalous dis- persions of polynucleotides in aqueous solution	27
Chapter IV. Principle and theory of the ellipsoid method	33
Chapter V. Sources of error in the ellipsoid method	37
A. Galvanic polarization	37
B. Adsorption	40
C. The moving system	41
D. Heat convections and mechanical disturbances	41
E. Voltage variations and distortion	43
F. Effect of the heterogeneity of the alternating field	45
Chapter VI. The apparatus	48
A. General arrangement of the apparatus	49
B. Suspension device for the moving system	51
C. The moving system	52
D. The electrode vessel	54
E. Reading device	55
F. High frequency sources	55
G. Frequency determination	60
H. Voltage measuring	60
Chapter VII. Measuring technique and precision	61

	Page
Chapter VIII. Control of the method	64
A. Continuous control	64
B. Measurements at different frequencies	66
C. Control with solutions of glycine	67
D. Control with solutions of urea	69
E. Control with solutions of potassium chloride	71
Summary, Part II	72
 <i>Part III. Experiments.</i> 	
Chapter IX. Some data regarding the polynucleotides examined	77
A. Previous investigations	77
B. Material	80
Chapter X. The dielectric constant of aqueous solutions of polynucleotides at different concentrations	83
A. Polyribose nucleotides	85
B. Polyribodesose nucleotides	87
C. Comments	89
Chapter XI. Anomalous dispersion of polyribodesose nucleo- tides in aqueous solution	91
Summary, Part III	96
General Summary and Conclusions	99
References	101

INTRODUCTION

Though the chemical composition of nucleic acids is now well known, we are still, in many respects, in the dark regarding their intramolecular bonds. Their chemical structure has been studied very extensively, but, as these investigations fall outside the scope of this work, it may suffice to refer the reader to Levene and Bass' monography »Nucleic Acids» (1931) and to the summaries compiled by BREDERECK (1938), GULLAND (1938) and ALLEN (1941).

The characteristic features of the polynucleotides are best reflected by their physico-chemical properties, which have been studied with various methods, in many cases requiring a very special and highly developed technique. That dielectric studies of polynucleotides should not previously have been made seems rather remarkable, seeing that, for the determination of dielectric constants at low frequencies, serviceable methods, which might yield valuable results, have long existed. Much more, however, could be gained by studies made at different high frequencies, as it will then be possible to determine important physical constants and to secure otherwise unobtainable information regarding the electrical symmetry and other properties of the substances. In regard to proteins, interesting results have been obtained by dielectric constant measurements at different frequencies within the radio frequency range; thus it should also be possible, by studies

of the dielectric properties, to gain greater knowledge of the polynucleotides. For the purpose of the present study, the author has collected primary dielectric data in regard to polyribodesose nucleotides from the thymus, and polyribose nucleotides from the pancreas.

As regards polynucleotides in aqueous solution, the determination of dielectric constants involves special difficulties, in view of their relatively high electric conductivity. The methods usually employed being found unsuitable for the purpose, the author adopted, instead, the so-called ellipsoid method, although it had proved to be effective chiefly at low frequencies. The ellipsoid method, however, was considered to be relatively simple in execution and measuring technique, and could be partly based on previous observations. For determinations at different frequencies within the radio frequency range which should be of particular interest in respect of polynucleotides, no method or apparatus had been elaborated for solutions with a high electric conductivity, although such an apparatus would be of the greatest value for many substances other than polynucleotides. As regards the ellipsoid method, extension of the range of frequency seemed possible, but was expected to meet with technical difficulties which would perhaps prove to be insurmountable.

In planning this study, it was accordingly decided to begin by determinations of the dielectric constant at low frequencies, in order afterwards, if possible, to proceed to determinations at various radio frequencies. This plan was followed up, and the technique resulted in the production of an apparatus which

could be used for an electric conductivity of up to $3 \cdot 10^{-3}$ ohm $^{-1}$ cm $^{-1}$ in the testing solution, firstly at low frequencies and secondly at various radio frequencies within the intermediate and long-wave ranges and, regards lower conductivities (at most $2 \cdot 10^{-4}$ ohm $^{-1}$ cm $^{-1}$), also for certain frequencies within the short-wave range.

As regards the arrangement of the present work, Part I gives a general review of the possibilities and theoretical bases of dielectric determinations. Part II contains an account of the method adopted, with special regard to possible sources of error and control of results. In Part III the results of the determinations of polynucleotides are noted and are taken as a basis for certain conclusions.

Table of the principal symbols

c	concentration
D	10^{-18} e.s.u. (1 Debye)
d	density
e	base of Napier's logarithm system
F	torque
f	frequency in c/s, kc/s or Mc/s (1 Mc/s = 10^3 kc/s = $= 10^6$ c/s)
I	increment
k	$1.38 \cdot 10^{-16}$ ergs per degree, Boltzmann's constant
M	molecular weight
N	$6.023 \cdot 10^{23}$ Avogadro's number
P	polarization
r	refractive index
T	absolute temperature
t	time
V	electric field strength
v	specific volume
ϵ	DC = dielectric constant
ϵ_l	ϵ at low frequency at infinite distance from the critical wave-length
ϵ_h	ϵ at high frequency at infinite distance from the critical wave-length
κ	specific electric conductivity
λ	wave-length
λ_c	critical wave-length
μ	dipole moment
τ	time of relaxation
η	viscosity
ω	angular velocity = $2 \cdot \pi \cdot f$
ω_c	critical angular velocity

PART I
THEORY

CHAPTER I

Dielectric polarization

Dielectric phenomena are now fairly generally known, as dielectric measurements have been adopted in widely different fields. The knowledge of dielectric conditions has played an important part also in clinical medicine, as it forms the basis for short-wave and ultrashort wave treatment (see RAJEWSKY, 1938). As a rule, however, dielectric measurements are intended for the determination of dielectric constants (in the sequel abbreviated DC) and especially their variation with the frequency of the electromagnetic oscillatory motion. Although the determinations of these constants as regards physiological substances date chiefly from the last ten or fifteen years, they have already assumed a prominent place in biochemistry.

A dielectric examination of the substances in aqueous solution (with which we are always concerned here) presents special difficulties both in theory and in practice. ARRHENIUS (1940), in connection with the publication of his studies on carbon monoxide hemoglobin, has thoroughly discussed these problems. For detailed particulars on the subject, the reader is therefore referred to his work. In the following merely a brief review of the fundamental concepts will be given.

The *dielectric constant* for a substance may be defined as the ratio between the capacity of a condenser (1) when the condenser is filled with the substance in question and (2) when it is in a vacuum. It is also the ratio between the forces with which charged bodies attract one another (1) in a vacuum and (2) in the substance referred to. The attraction which a substance can exercise in this way is based on *polarization*, that is, the molecules in an electromagnetic field produce a field

system adjusted to the direction of the outer field. In this way the capacity of the condenser increases, whilst the force of attraction between the charged bodies diminishes. Polarization may take place within the entire electromagnetic spectrum, including the optic range. According to Maxwell's electromagnetic theory of light, $DC =$ the square of the ratio between the velocity of light in a vacuum and in the medium, *i.e.*

$$\epsilon = r^2 \quad (1)$$

where r is the index of refraction. This calculation applies only to an infinite wave-length, *i.e.* where the distance to the nearest absorption band is infinite. Practically speaking, however, it would suffice to use long-wave light. In distinction from normal dispersion, where the index of refraction changes slowly, anomalous dispersion, in which the index of refraction undergoes marked changes, is produced by all substances within certain limited areas, as a rule within the ultraviolet and infra-red spectral ranges. As regards anomalous dispersion within the optic range, the connection between the refractive index and DC is illustrated by Ketteler-Helmholtz formula

$$r^2 = \epsilon + \frac{C_1}{\lambda^2 - \lambda_1^2} + \frac{C_2}{\lambda^2 - \lambda_2^2} + \frac{C_3}{\lambda^2 - \lambda_3^2} + \dots \quad (2)$$

where ϵ denotes DC for the substance with the Hertz waves and $\lambda_1, \lambda_2, \lambda_3 \dots$ wave-lengths in the range of anomalous dispersion. $C_1, C_2, C_3 \dots$ are constants which are characteristic of the substance and in which the number of polarizable components is included. Thus for $\lambda = \lambda_1$, r will evidently be infinitely great, but, owing to absorption, the rise of the index will be finite.

A good idea of the way in which the dielectric constant is built up can be obtained by following the variation of DC with the frequency. Different anomalous dispersions are shown in Fig. 1. In area I, where the frequency is very high, corresponding to that of short-wave ultraviolet, DC passes through a minimum and a low maximum. The change is due to a shifting of the electron paths and entail the result that the

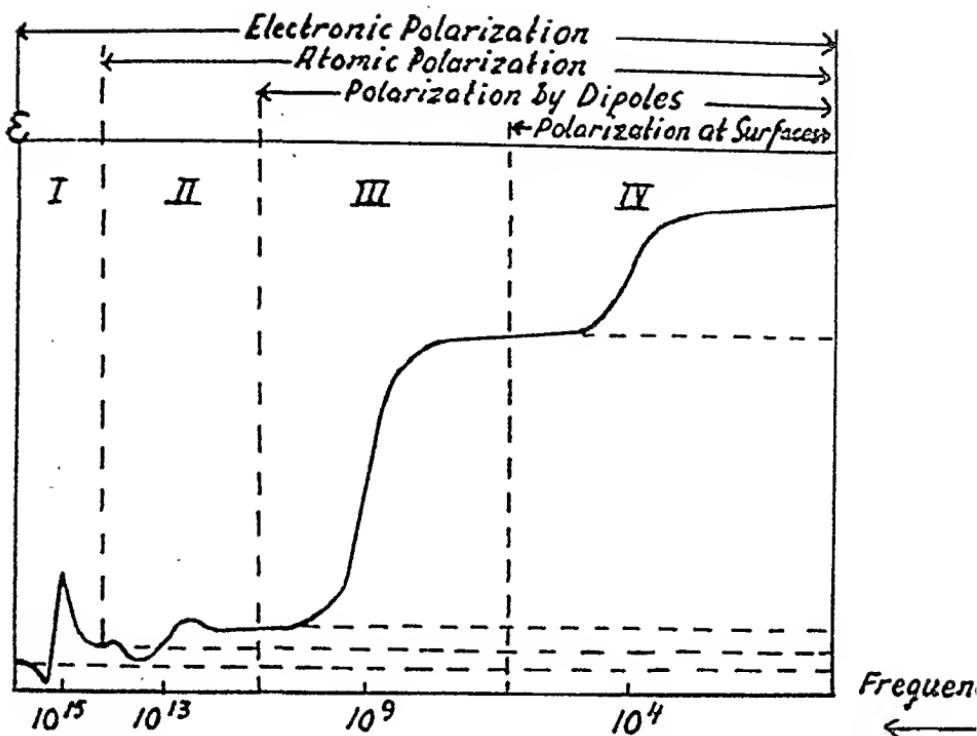


Fig. 1. Diagram of DC variation with the frequency.

For the sake of perspicuity, the ranges of frequency and the changes in DC are indicated merely approximately.

positive and negative centres of gravity no longer coincide, so that a *dipole* is produced. If the distance between the centres is l and the sum of the positive charges E (the sum of the negative charges $= -E$) we obtain the *dipole moment*, μ , according to

$$\mu = E \cdot l \quad \text{e.s.u.} \quad (3)$$

The polarization — measured in electric moment per unit of volume —, thus produced is termed *electronic polarization*. At lower frequencies, but still within the optic range (corresponding to area II in Fig. 1), DC undergoes similar changes with the frequency as before. The oscillations are now slower and, besides the electrons, the protons are also displaced. The distance between the positive and negative centres of gravity is now increased, and thus also the dipole moment. This in-

crease in polarization is termed *atomic polarization* and occurs as a rule in the range of infra-red light.

The said polarizations are produced under the action of an outer field and are therefore said to be *induced*. But molecules or molecule radicals, even in an electroneutral state, often have different negative and positive centres of gravity (*permanent dipoles* according to Debye). Owing to their larger mass, they cannot contribute to the polarization at the higher frequencies in area I and II. On the other hand, at a lower frequency they orient in the field, overcoming thermal agitation and friction to adjacent molecules, thus giving rise to *polarization by orientation* (area III in Fig. 1). It occurs at different frequencies varying with the size of the mobile parts, as a rule between 10^{12} — 10^4 c/s. Moreover, yet another form of polarization occurs at boundary surfaces (area IV in Fig. 1). This *boundary-surface polarization*, though of importance in the study of biological tissues (heterogeneous media), has no bearing on the present study.

By far the most important of the said polarizations, from the present point of view, is polarization by orientation. Within its wave-length range, about 3 cm—30 000 m, several different dispersions can be found. The conditions in this respect for an aqueous solution of substances with a high molecular weight are shown schematically in Fig. 2. For water the anomalous dispersion lies at about 10^{10} c/s, corresponding to a wave-length of about 0.03 metre (I). At longer wavelengths, therefore, the water will not directly affect the determination of the dispersions of substances with a higher molecular weight. Water has, however, a very marked polarization, which is liable to be modified by dissolved substances, etc. It cannot be taken for granted that dipoles dissolved in water will cause a rise of its DC. This is the fact only as regards substances with large dipole moments. As for substances with small dipole moments, their increase in polarization may be completely counterbalanced by a fall in the polarization of the water. Also as regards substances in very dilute solutions, it

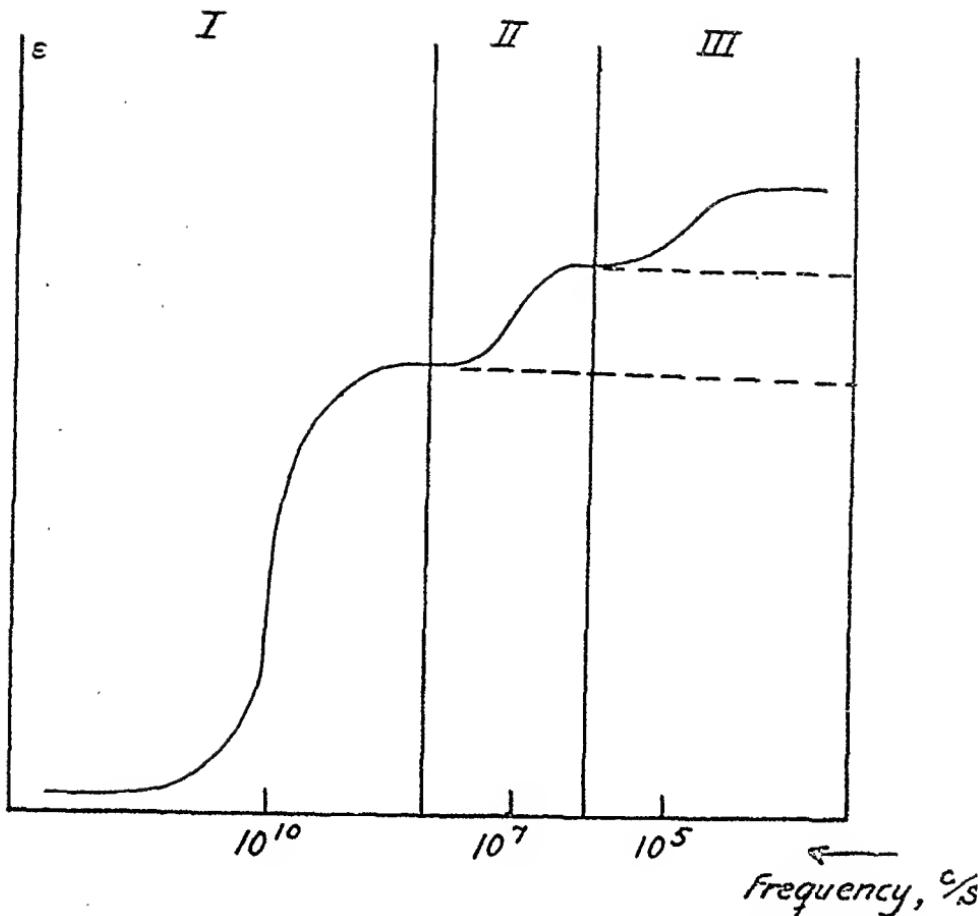


Fig. 2. Diagram of DC variation with the frequency for aqueous solution of a high-molecular substance.

The substance is assumed to increase the polarization of the water. For the sake of perspicuity, the dispersion ranges are widely separated and merely approximate.

must be assumed that changes in DC are primarily due to a change in the polarization of the water. On the other hand, it is likely that a dipole with a high moment — besides its contribution to the polarization by the orientation of dipoles —, may decrease the association of the water, so that its polarization increases. Thus — though indirectly — the dipole moment will nevertheless be a function of the DC of the water.

The other dispersions indicated in Fig. 2 may be, for example, polarization by orientation of a radical (II) or an entire

molecule (III). If a molecule has electric symmetry in two planes, however, it may give rise to two dispersions. This would give a similar curve, in which the dispersion within area II would be produced by the rotation of the molecule round its long axis and, at lower frequencies, round its short one (III).

That the dispersion takes place at different frequencies, is due to the fact that it takes more time for a dipole to orient itself in a field, the longer its axis. The time required by a dipole to make one revolution round its axis is termed the *time of relaxation* and stands in a certain ratio to the length of the dipole. In all dispersions the increase of DC with diminishing frequency has a typical and mathematically defined course, the so-called *anomalous dispersion curve*. Its middle-point corresponds to a frequency termed the *critical frequency* (or the *critical wave-length*). By determining DC at different frequencies, this curve can be constructed and the corresponding mathematical function determined. From this the dipole moment and the time of relaxation can be computed, and an idea of the size of the rotating units can be obtained. This may be said to be the aim of dielectric studies of this nature. The theoretical difficulties involved in the calculations will be discussed in the following chapter.

CHAPTER II

Computation of dipole moment and time of relaxation

Clausius-Mosotti's relation for the molar polarization, P_r , reads

$$P_r = \frac{M}{d} \cdot \frac{r^2 - 1}{r^2 + 2} \quad \text{cm}^3 \quad (4)$$

where M is the molecular weight, r the index of refraction and d the specific weight. The molar polarization is a measure of the volume of the molecules in a substance and, according to Mosotti's theory of dielectrics, is a universal material constant for the substance, and independent of pressure, temperature and state of aggregation. The molar polarization, however, will be quite different for many substances according as it is computed from the refractive index or from DC with Hertz waves (from $\epsilon = r^2$), and shows distinct dependence on the temperature. An explanation of the deviations was given by Debye's theory of the permanent dipole moment (see his monography »Polare Moleküle«, Leipzig 1929). According to Debye, the molar polarization, computed from the index of refraction, increases at long wave-lengths owing to the polarization by the orientation of permanent dipoles. The polarization at very long wave-lengths, P_l , will then be

$$P_l = \frac{M}{d} \cdot \frac{\epsilon_l - 1}{\epsilon_l + 2} = P_r + P_o \quad \text{cm}^3 \quad (5)$$

where P_o is the polarization of orientation and ϵ_l the dielectric constant measured. For the permanent dipole moment, μ ,

Debye derived the following connection with P_o

$$P_o = \frac{4 \cdot \pi \cdot N}{9 \cdot k \cdot T} \cdot \mu^2 \quad \text{cm}^3 \quad (6)$$

where N is Avogadro's number, k Boltzmann's constant and T the absolute temperature.

From the equations (4), (5) and (6), the dipole moment for a homogeneous substance can be estimated if we know the molar polarization on both sides of, and far from, the critical wave-length. For a solution in a *non-polar* solvent, such as benzene, cyclohexane, etc., Lorenz-Lorentz' summation formula can be used for determining the polarization by orientation of the substance. From this the dipole moment can then be calculated. According to the summation formula, the polarization (P_{12}) for a mixture is composed of the molar polarizations of the component substances in proportion to their mole fractions, that is,

$$P_{12} = m_1 \cdot P_1 + m_2 \cdot P_2 \quad \text{cm}^3 \quad (7)$$

where m_1 and m_2 are mole fractions of substances with the molar polarizations P_1 and P_2 . For a dilute solution of a substance in a solvent with the polarization P_M , the polarization, P_L , of the solution will be

$$P_L = P_M + c \cdot P_S \quad \text{cm}^3 \quad (8)$$

where P_S is the polarization of the dissolved substance at the molar concentration c . For non-polar solvents, the polarization at frequencies corresponding to that of long-wave light and an electrostatic field is practically the same; but, as regards the solution of a substance with a permanent dipole moment, a difference will arise owing to orientation polarization, P_{SO} . It can then be shown that

$$c \cdot P_{SO} = \frac{M}{d} \left(\frac{\epsilon_l - 1}{\epsilon_l + 2} - \frac{r^2 - 1}{r^2 + 2} \right) \quad \text{cm}^3 \quad (9)$$

where for a solution with the molar concentration c , r is determined for long-wave light and ϵ_l at a frequency which is very low (really an electrostatic field).

From the formula (9) we obtain, with the use of equation (6)

$$c \cdot P_{so} = \frac{M}{d} \left(\frac{\epsilon_l - 1}{\epsilon_l + 2} - \frac{r^2 - 1}{r^2 + 2} \right) = c \cdot \frac{4 \cdot \pi \cdot N}{9 \cdot k \cdot T} \cdot \mu^2 \text{ cm}^3 \quad (10)$$

which gives

$$\mu = 0.0128 \cdot 10^{-18} \sqrt{\frac{M \cdot T}{c \cdot d} \left(\frac{\epsilon_l - 1}{\epsilon_l + 2} - \frac{r^2 - 1}{r^2 + 2} \right)} \text{ e.s.u.} \quad (11a)$$

If the critical frequency corresponds to wave-lengths at a great distance from the optical range, instead of the refractive index, the dielectric constant at high frequencies, ϵ_h , can be determined and used. The dipole moment will then be

$$\mu = 0.0128 \cdot 10^{-18} \sqrt{\frac{M \cdot T}{c \cdot d} \left(\frac{\epsilon_l - 1}{\epsilon_l + 2} - \frac{\epsilon_h - 1}{\epsilon_h + 2} \right)} \text{ e.s.u.} \quad (11b)$$

The computation of the dipole moment is based on several assumptions, more especially the validity of Clausius-Mosotti's formula. In its derivation it is assumed that the molecules have a globular shape. One might then have expected that it would be devoid of general validity. Nevertheless it has been found to be applicable not only to gases and cubic crystals, but also to many dilute solutions in non-polar solvents. An inference from Clausius-Mosotti's relation is that the summation formula should be applicable, *i.e.* that as regards a solution, the polarization should increase proportionally to the concentration of the dissolved substance. This, however, is not invariably the case. A useful value can nevertheless often be obtained by extrapolating to infinite dilution, in which case the procedure indicated by HEDESTRAND (1929) will be found valuable.

In *polar* solvents, owing to the intense interaction between the molecules, Clausius-Mosotti's relation is not applicable. The expression for the molar polarization in Debye's for-

mula will therefore be incorrect and, as regards solvents in polar media, the formula will not give dipole moments corresponding with the estimated distance between the charges.

Theoretical difficulties in computing the dipole moments for substances in polar solvents are set off by simple empirical relations. For solutions of substances in *e. g.* water, we often find, within an extended range of concentration, that

$$\varepsilon_s = \varepsilon_M + c \cdot a \quad (12)$$

where ε_s is the DC of a solution in a medium with $DC = \varepsilon_M$, c the molar concentration, and a is a constant characteristic of the substance. If DC increases with the concentration, the factor a is termed »increment» (in the sequel abbreviated I), otherwise »decrement».

J. WYMAN JR (1936), who has set up important empirical relations in this respect, considers that a constant increment for different concentrations signifies that the Lorenz-Lorentz' formula (7) is applicable. He states that the dipole moment can then be computed for solutions in polar media, and sets up an empirical formula for the molar polarization, P ,

$$P = \frac{M}{d} \cdot \frac{\varepsilon + 1}{g} \quad \text{cm}^3 \quad (13)$$

where g is a constant which varies in value for different groups of substances. With the use of ε_l and ε_h for such a solution, it will be found, similarly as before, that

$$\mu = 0.0128 \cdot 10^{-18} \sqrt{\frac{M \cdot T}{c \cdot d} \cdot \frac{\varepsilon_l - \varepsilon_h}{g}} \quad \text{e.s.u.} \quad (14)$$

Thus here too the computation is based on Debye's formula, and it is only the expression for the molar polarization that differs. On the basis of comparisons with a large number of substances, WYMAN put $g = 8.5$. In many cases the deviations were considerable, and he tried also the values 6.2 and 11. For amino-acids ONCLEY (1938) found $g = 5.8$.

Besides convincing experimental evidence, WYMAN's views have received theoretical support from the theory advanced

by ONSAGER (1936). But, as ONSAGER's calculations relate to spherical molecules, where the charges are supposed to be placed in the centre — which certainly does not apply to poly-nucleotides —, they will not be summarized here.

As ARRHENIUS (1940) has pointed out, WYMAN's polarization formula must be regarded as a specific case of a general formula with unknown functions, which are constant only within groups of very analogous substances. Within such a group, relative dipole moments computed from dilute aqueous solutions can be used, and it is of minor importance on what polarization formula the calculation is based. Practically speaking, it is only the absolute magnitude of the moments that is different.

Summing up, it may be stated that there is no generally valid formula for polarization in aqueous solution. The empirical formula can be used only if some substance suitable as a basis of comparison is well known. For the computation of relative dipole moments, Debye's formula is serviceable.

In order to estimate the dipole moment, it will be necessary — as shown above (formula 11 b) —, to know the molar polarization of the solution at frequencies at a long distance above and below the critical frequency. This, however, presupposes a knowledge of DC at the frequencies in question. These DC values may either be directly determined, or else computed from the anomalous dispersion curve, if it can be experimentally determined.

The anomalous dispersion proceeds according to the function

$$\epsilon_{\omega} = \epsilon_h + \frac{\epsilon_l - \epsilon_h}{1 + \left(\frac{\epsilon_l + 2}{\epsilon_h + 2} \right)^2 \cdot \tau_D^2 \cdot \omega^2} \quad (15)$$

where ϵ_{ω} is DC at the angular velocity ω and τ_D the time of relaxation according to Debye. The function contains three unknowns ϵ_l , ϵ_h , and τ_D , and is mathematically unwieldy. It is therefore advisable to write it under the formula

$$\left(\frac{\varepsilon_l + 2}{\varepsilon_h + 2}\right)^2 \cdot \tau_D^2 = \frac{1}{\omega^2} \cdot \frac{\varepsilon_l - \varepsilon_\omega}{\varepsilon_\omega - \varepsilon_h} \quad (16)$$

and to determine ε_l , ε_h and τ_D by trying with assumed values of ε_l and ε_h until the left member does not show any systematic deviation at different frequencies.

From the anomalous dispersion curve, the time of relaxation is thus obtained, according to Debye. In this way the molecular weight of the unit rotating in the electric field can be computed. According to Debye, the time of relaxation is an expression of the resistance opposed by adjacent molecules, against the orientation of the dipole. Proceeding from Stoke's calculations for the friction on the rotation of a ball in a medium with the viscosity η , Debye derived for a globular molecule, with the radius a , an expression for the time of relaxation, τ_D

$$\tau_D = \frac{4 \cdot \pi \cdot a^3 \cdot \eta}{k \cdot T} \quad \text{sec.} \quad (17)$$

From the equation (15), τ_D is obtained by finding the central point of the curve (at the critical frequency ω_c), where

$$\frac{\varepsilon_l - \varepsilon_\omega}{\varepsilon_\omega - \varepsilon_h} = 1$$

Then

$$\tau_D = \frac{1}{\omega_c} \cdot \frac{\varepsilon_h + 2}{\varepsilon_l + 2} \quad \text{sec.} \quad (18)$$

For a spherical molecule with the radius a , the molecular weight, M , is

$$M = \frac{N}{v} \cdot \frac{4 \cdot \pi \cdot a^3}{3} \quad (19)$$

where v is the specific volume and N Avogadro's number. The equations (17) and (19) give

$$M = \frac{N}{v} \cdot \frac{k \cdot T \cdot \tau_D}{3 \cdot \eta} \quad (20)$$

If the electric axes are different in two planes, the orientation in the electric field can be effected by rotation either

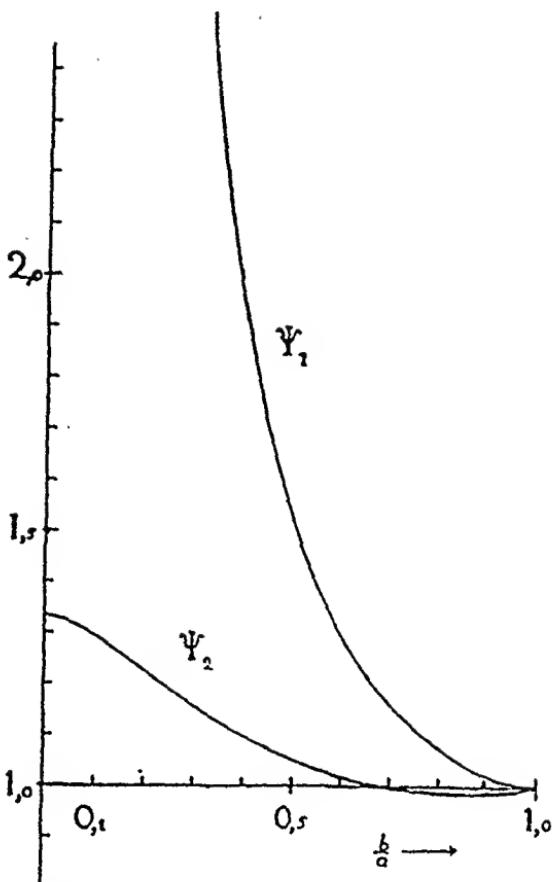


Fig. 3. Perrin's form factors for rotation ellipsoids (after Arrhenius).

Ψ_1 for rotation round the short axis, b ,
 Ψ_2 for rotation round the long axis, a .

round the long axis (a) or the short one (b). The found time of relaxation will be different in relation to τ_D . On the rotation of the molecule round the short axis we obtain a different time of relaxation, τ_1 , than that computed according to Debye. Then

$$\tau_1 = \Psi_1 \cdot \tau_D \quad \text{sec.} \quad (21 \text{ a})$$

and on rotation round the long axis, correspondingly

$$\tau_2 = \Psi_2 \cdot \tau_D \quad \text{sec.} \quad (21 \text{ b})$$

ψ_1 and ψ_2 are form factors indicated by PERRIN (1934). As shown in Fig. 3 (reproduced from ARRHENIUS' work, 1940),

they vary for different axis ratios in the molecule regarded as a rotation ellipsoid. Here it is of interest to note that for oblong rotation ellipsoids, ψ_2 with increasing asymmetry moves towards a limit value $= \frac{4}{3}$, and that the form factor is but little affected by minor variations in the axis ratio. For example, if $b/a = \frac{1}{5}$, the deviation from $\psi_2 = \frac{4}{3}$ will be less than 10 %, and for $b/a = \frac{1}{10}$ less than 3 %. Thus, for thread molecules rotating round the longer axis, the determination of the molecular weight is practically independent of changes in the form factor. The required formula for the determination of the molecular weight, with the use of the equations (20) and (21 b), will be

$$M = \frac{N}{v} \cdot \frac{k \cdot T \cdot \Psi_2 \cdot \tau_D}{3 \cdot \eta} = \frac{N \cdot k \cdot T \cdot \Psi_2}{3 \cdot v \cdot \eta \cdot \omega_c} \cdot \frac{\epsilon_h + 2}{\epsilon_l + 2} \quad (22)$$

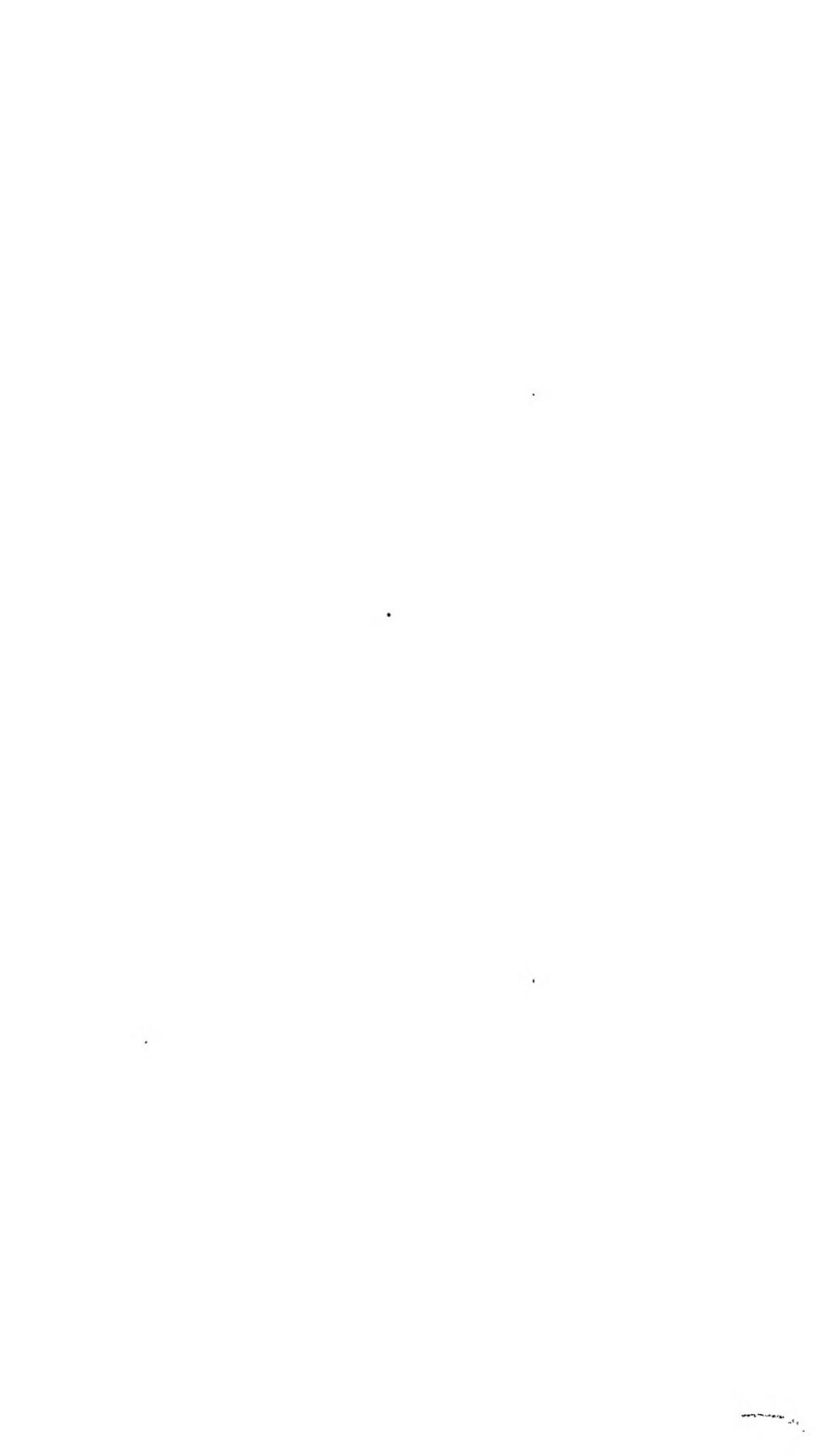
If the critical wave-length λ_c is introduced, according to the formula $\lambda_c = \frac{2 \pi \cdot \bar{c}}{\omega_c}$ (\bar{c} = velocity of light $= 3 \cdot 10^{10}$ cm sec. $^{-1}$), and $\psi_2 = \frac{4}{3}$, we obtain for an aqueous solution at 20° C. ($T = 20^\circ + 273^\circ$, $\eta = 0.10$, $k = 1.38 \cdot 10^{-16}$, $N = 6.02 \cdot 10^{23}$)

$$M = \frac{N \cdot k \cdot T \cdot \Psi_2 \cdot \lambda_c}{3 \cdot v \cdot \eta \cdot 2 \pi \cdot \bar{c}} \cdot \frac{\epsilon_h + 2}{\epsilon_l + 2} = 5.7 \cdot \frac{\lambda_c}{v} \cdot \frac{\epsilon_h + 2}{\epsilon_l + 2} \quad (23)$$

It should be noted that λ_c is then reckoned in cm. The molecular weight is thus determined from dielectric data (and the specific volume).

Although there are several methods for computations of molecular weights in high-molecular substances, determination by dielectric investigation is of special interest. It determines the molecular weight of the units which orient, independently of one another, in an electric field. Only if the molecule rotates in its entirety, will the molecular weight correspond to that computed with other methods. Dielectric studies thus happily serve to supplement other methods for the computation of molecular weights.

PART II
METHOD



CHAPTER III

The possibilities of determining anomalous dispersions of polynucleotides in aqueous solution

For dielectric investigations, several different methods are available. Obviously, however, the technique and apparatus must be adapted to the special requirements involved in the nature of the problem.

It was impossible to foresee the magnitude of the changes in the polarization of the water that would be entailed by the polynucleotides, or what concentrations would have to be examined. A comparison with proteins having high molar increments naturally suggested itself. They are usually examined in concentrations of a few grams per 100 ml, and then show an increase of a few units as compared with the DC of the water. Though it was presumed that the nucleic acids could be examined in considerably weaker solutions, the first essential, in view of the electrolytic properties of those acids, was obviously that the method should permit a comparatively high electric conductivity. At a concentration of 0.5 g/100 ml, the specific conductivity for the sodium salt of polyribodesose nucleotide is about $4 \cdot 10^{-4}$ ohm $^{-1}$ cm $^{-1}$ and for that of the polyribose nucleotide, at pH 6, about $7 \cdot 10^{-4}$ ohm $^{-1}$ cm $^{-1}$. For a comparison with mononucleotides that might be of interest in this connection, the requirements would be considerably higher. Thus, for the sodium salt of uridylic acid in a concentration of 0.5 100/ml, the specific conductivity at pH 7.3 is about $2.4 \cdot 10^{-3}$ ohm $^{-1}$ cm $^{-1}$.

Under the above indicated conditions, the methods which come up for consideration may be arranged under the following groups:

- 1) wave-length measuring methods,
- 2) capacity measuring methods, and
- 3) force methods.

1. *Wave-length measuring methods.*

Drude (1893) showed that it was possible to use standing waves of high-frequency oscillations, produced on Lecher wires. By computing the maxima and minima of the amplitude, the wave-length could be directly measured. DC is then obtained from the equation

$$\frac{\epsilon_x}{\epsilon_{vacuum}} = \frac{\lambda_x^2}{\lambda_{vacuum}^2}$$

Though this method admits of relatively high electric conductivity, its exactitude diminishes as the conductivity increases, owing to gradual flattening of the amplitude maxima. It has, however, been adopted by DRÁKE, PIERCE and Dow (1930), and it seems to be of value. It should be noted, however, that it can be used only at high frequencies.

2. *Capacity measuring methods.*

By comparing the capacity of a condenser filled with a substance of known DC with its capacity when filled with the testing solution, DC is obtained. The determination of the capacity can be made either (1) with a suitably modified Wheatstone's bridge, or (2) by utilizing the principle of an alternating-current resonance in a circuit to which the condenser is connected.

The *bridge method* has great advantages. The losses in the condenser due to the testing solution can be largely eliminated, and the method can be used for fairly high conductivities e.g. $6 \cdot 10^{-5}$ ohm $^{-1}$ cm $^{-1}$ in Arrhenius' apparatus of 1940). The range of frequency is large. Though measurements at

wave-lengths under 100 m present great difficulties, successful attempts have been made to extend the range of measurements downwards (30—40 m COLE and CURTIS 1937, 50 m ARRHENIUS 1944). Upwards, the wave-length range extends to 6 000 m and over.

The second-mentioned group of capacity-measuring methods, which may be collectively termed *resonance methods*, are based on the determination of the resonance. They have been widely adopted for *non-conductive* solutions, for which purpose they are by far the most exact. But where the testing solution has a marked conductivity, the various applications of this principle are all marred by serious drawbacks. Firstly, there will be a considerable loss in precision, and secondly the resonance will shift in a way that eludes control. The error can be shown to be proportional to the square of the conductivity and the square of the wave-length. Several methods intended to allow for the electric conductivity by comparisons with solutions of strong electrolytes have been elaborated (WALDEN, ULIICH and WERNER 1925, GRAFFUNDER and WEBER 1931, WILLIAMS and ELLIOT 1939, etc.). These efforts to extend the facilities for measurement have not been very successful. As regards measurements at different frequencies, such methods are serviceable only at wave-lengths of less than 400 metres. For a specific electric conductivity of 10^{-4} ohm $^{-1}$ cm $^{-1}$, GRAFFUNDER and WEBER (1931) had to make a correction of over 10 per cent.

3. Force methods.

There are several different force methods, but, practically speaking, only two of them can be contemplated here, notably the electrometer and the ellipsoid methods. It should first be noted that, at any rate in theory, these methods are independent of electric conductivity, whence their use in electrically conductive solutions is limited merely by secondary effects due to heating.

The electrometer method. This method is based on the principle of an electrometer filled with a testing solution. It has been developed especially by CARMAN (1924—1929) and his associates. In the adopted by CARMAN, YOUNG and SMITH (1929), the moving system consists of two platinum plates with curved surfaces. The plates are interconnected and are suspended by a thin string. Each of them dips into a glass vessel with similar, but firmly fixed, plates. A control solution (water) is introduced into the one vessel, the testing solution into the other. The electrodes in each vessel are subject to different alternating voltages, which are varied until the attractive forces neutralize one another. The DC of the solutions will then be in inverse proportion to the squares of the voltages. In order to avoid polarization, an alternating current of 500 c/s is used for the conductive testing solution, and a current of 50 c/s for the control solution.

A moving system of the large required bulk, in conjunction with the considerable liquid resistance, evidently necessitates the use of high field strengths. Experience also shows that the difficulties entailed by the heating of the testing solution are very marked. Even if it was cooled, the application of the method was greatly restricted, and the results were shifting and uncertain.

This method, however, has certain distinct advantages. By basing all measurements on comparison with a standard solution, it can serve as a null method. In theory, it is completely independent of the frequency. The upward range of frequency seems to be limited only by difficulties of execution.

NERNST (1896) has pointed out that the different flux of the lines of force between electrodes with different DC involves a theoretical error. In the above indicated execution of the method, however, this seems to be of minor importance, relatively to the incidental errors which actually occur.

The ellipsoid method. This being the method adopted in the present study, its principle and execution in practice call for further explanation. As a more detailed account of it is given in

the following chapters, it need only be noted here that in this method one measures the torque exercised on a rotation ellipsoid of small dimensions in an electric field. This method has the outstanding merit that the moving system is lighter and the required field strength considerably lower than in the electrometer method. As regards a metallic ellipsoid, the method is theoretically independent of the electric conductivity of the solution and the frequency (see Chapter IV). The results obtained in the application of the ellipsoid method are decidedly encouraging, especially when it is used at low frequency, in which case solutions with a specific electric conductivity of more than 10^{-3} ohm $^{-1}$ cm $^{-1}$ can be examined.

The choice of method for this study of polynucleotides could be based only on theoretical considerations. One could not know how strong solutions would have to be used, nor how high a conductivity the method must permit. Nor was the range of the anomalous dispersion known, and in that regard there were widely different possibilities, as the times of relaxation would vary considerably according to the way in which the polynucleotides rotated, *i. e.*, whether the entire molecule took part in the polarization or, for example, isolated mononucleotides. For a thread-like molecule rotating as a unit in an alternating-current field and having a molecular weight of 200 000 (as found for sodium thymo-nucleinate in dilute solution), the dispersion may be estimated to fall mainly between 100 and 3 000 m. If, on the other hand, the rotating units were very minute (*e. g.* mononucleotides), the dispersions might occur at a wave-length of a metre or so.

The previously elaborated method that covers the major part of this wave-length range is the bridge method. But, as it did not permit measurements at a higher specific electric conductivity than at most 10^{-4} ohm $^{-1}$ cm $^{-1}$, it could not *a priori* be regarded as satisfactory.

The methods which admit of sufficiently high conductivity are, as shown above, firstly the wave-length measuring and certain resonance methods, and secondly the force methods. The wave-length measuring and resonance methods, however,

can be used only within a narrow range with very short wave-lengths, which, as regards high-molecular substances, is *prima facie* scarcely desirable.

With a view to a suitable wave-length range, it seemed that only the *force methods* could be seriously contemplated. The choice between the electrometer and the ellipsoid methods was determined by practical considerations, and the ellipsoid method was found to be distinctly preferable. The results of this investigation, however, show that, in view of the immensely high increments shown by the polynucleotides, also other methods may be adopted. Despite the extended range of measurement given to the ellipsoid method in its new form, the question of the choice of method for the study of polynucleotides at the highest frequencies still remains to be settled. In short, a complete dielectric study of polynucleotides cannot be made with a single method.

CHAPTER IV

Principle and theory of the ellipsoid method

An asymmetrical body in a dielectric with negligible conductivity is polarized under the action of an electric field and strives to adjust itself in the field direction. Mathematical computation of the torque when the body has the form of a rotation ellipsoid in a non-conductive medium was made at an early date and forms part of the classical electromagnetic theory. But where the electrical conductivity is not negligible, the conditions become more complicated. The introduction of the *ellipsoid method* by FÜRTH (1924) must therefore be regarded as a great advance.

FÜRTH found the following relations: Let it be supposed that a rotation ellipsoid with the specific electric conductivity κ_0 , and having the axis of rotation of the length a and the other axis of the length b , is suspended by a thin wire in a medium with the electric conductivity κ and with $DC = \varepsilon$, so that the axis of rotation is horizontal. In a homogeneous electric field (field strength V), where the lines of force run horizontally, forming a angle α with the axis of rotation, the ellipsoid will then be given a torque, F . According to the relative size of the axes, we then obtain the following cases:

If a is $\gg b$, then

$$F = \varepsilon \cdot V^2 \cdot \frac{a^3}{105} \cdot \frac{3\frac{\kappa_0}{\kappa} - 4}{\frac{\kappa_0}{\kappa} + 1} \cdot \frac{1}{\frac{\kappa_0}{\kappa} - 1} + \frac{b^2}{a^2} \cdot \ln \frac{2}{\frac{b}{a} \cdot e} \cdot \sin 2\alpha \quad (24a)$$

If a is $\ll b$, then

$$F = -\varepsilon \cdot V^2 \cdot \frac{2b^4}{105a} \cdot \frac{3 + 4 \cdot \frac{\kappa_0}{\kappa}}{\frac{\kappa_0}{\kappa}} \cdot \frac{1}{\frac{4}{\frac{\kappa_0}{\kappa} - 1} + \frac{\pi \cdot a}{b}} \cdot \sin 2\alpha \quad (24b)$$

The torque evidently has a maximum for $\sin 2\alpha = 1$. Thus, on measuring the torque, the axis of rotation should form an angle of 45° to the outer field.

For an ellipsoid of platinum ($\kappa_0 \approx 10^5 \text{ ohm}^{-1}\text{cm}^{-1}$) in a dilute electrolyte solution, where $\frac{\kappa_0}{\kappa}$ is large, the formulae are simplified.

For $a \gg b$, we obtain

$$F = \varepsilon \cdot V^2 \cdot \frac{a^3}{105} \cdot \frac{3}{\frac{b^2}{a^2} \ln \frac{2}{\frac{b}{a} \cdot e}} \cdot \sin 2\alpha = C_1 \cdot V^2 \cdot \varepsilon \cdot \sin 2\alpha \quad (25a)$$

and for $a \ll b$

$$F = -\varepsilon \cdot V^2 \cdot \frac{2b^4}{105a} \cdot \frac{4}{\frac{\pi \cdot a}{b}} \cdot \sin 2\alpha = C_2 \cdot V^2 \cdot \varepsilon \cdot \sin 2\alpha \quad (25b)$$

where C_1 and C_2 are constants dependent on the material and dimensions of the ellipsoid. When the angle of torque, β , for a wire of a certain material, and of a certain length and thickness is proportional to F , we obtain for small angles

$$\beta = C_s \cdot \varepsilon \cdot V^2 \quad (26)$$

and thus

$$\frac{\beta_x}{\beta_s} = \frac{\varepsilon_x}{\varepsilon_s} \quad (27)$$

where the indices x and s relate to the testing solution and the standard solution (e.g. water), respectively.

The relation between the deflexion and DC applies, strictly speaking, only to rotation ellipsoids. Deviations from the ellipsoid shape render this relation dependent, *inter alia*, on the DC (which is not known) of the ellipsoid material. In FÜRTH's derivation of the formulae (24), all the terms in which the DC of the ellipsoid material was involved were ruled out, for reasons of symmetry. FÜRTH pointed out that the ellipsoid is the only geometrical body which — owing to its having a homogenous internal electric field —, has this property. His disciple HOLLÄNDER (1930) studied the conditions for a ring with circular cross section and found that, as regards certain dimensions, a simple relation for the torque could be obtained, in which, however, the DC of the ring material was included. The constant, however, could be determined in that way.

COHN (1931) contended that the simple formula (26), might be applicable irrespective of the ellipsoid shape. This supposition, however, seems to be valid only where the ellipsoid has a stationary position.

When the deflexion is proportional to the square of the field strength, it is independent of the direction of the current, and alternating voltage can be employed. In that case the effective value can be used for V . FÜRTH considered that almost any frequency could be taken, the range of frequency being limited only by the requirement of a »quasi-stationary field», *i.e.* a wave-length which is long relatively to the ellipsoid. The frequency, however, must be so high that galvanic polarization is avoided.

BJÖRNSTÅHL (1941), in a critical study of the ellipsoid method theory, pointed out that it was incorrect to infer from FÜRTH's formulae that the method was independent of the frequency. Taking the frequency into account, he made general computations for the torque and found the following relations:

$$F = \frac{1}{4} \cdot V_0^2 \cdot v' \cdot (A - B) \cdot \left\{ \varepsilon_0 [(a' + A)(a' + B) - (b')^2] - \frac{4\pi \cdot b' \cdot \kappa}{\omega} (2a' + A + B) \right\} \cdot \frac{[(a' + A)(a' + B) - (b')^2]^2 + (b')^2 (2a' + A + B)^2}{[(a' + A)(a' + B) - (b')^2]^2 + (b')^2 (2a' + A + B)^2} \cdot \sin 2\gamma \quad (28)$$

where

$$a' = \frac{\kappa(u_0 - u) \cdot 16 \cdot \pi^2 + \varepsilon \cdot \omega^2 (\varepsilon_0 - \varepsilon)}{(u_0 - u)^2 \cdot 16 \cdot \pi^2 + \omega^2 \cdot (\varepsilon_0 - \varepsilon)^2}$$

$$b' = \frac{4 \cdot \pi \cdot \omega \cdot [\varepsilon(u_0 - u) - u(\varepsilon_0 - \varepsilon)]}{(u_0 - u)^2 \cdot 16 \cdot \pi^2 + \omega^2 \cdot (\varepsilon_0 - \varepsilon)^2}$$

$$A = \frac{1 - (e')^2}{(e')^2} \cdot \left[\frac{1}{2 \cdot e'} \ln \frac{1 + e'}{1 - e'} - 1 \right] \text{ if } a > b$$

and

$$A = \frac{1}{(e')^2} \left[1 - \frac{\sqrt{1 - (e')^2}}{e'} \arcsin e' \right] \text{ if } a < b$$

$$B = \frac{1 - A}{2}$$

γ = the angle between long axis of the ellipsoid and the lines of force of the field

ε_0 = DC of the ellipsoid material

e' = eccentricity of the ellipse which, by rotation, gives the ellipsoid

v = the volume of the ellipsoid

V_0 = the peak value of the alternating voltage

The formula is rather cumbersome, but, if $\frac{\kappa}{u_0}$ is small (as in metallic ellipsoid material), $a' \approx 0$ and $b' \approx 0$.

BJÖRNSTÅHL thus obtained

$$F = \frac{V_0^2 \cdot v}{4} \cdot \varepsilon \cdot \frac{A - B}{A \cdot B} \cdot \sin 2\gamma \quad (29)$$

of which

$$F = C_4 \cdot V_0^2 \cdot \varepsilon \cdot \sin 2\gamma \quad (30)$$

i.e. practically the same relation as that found by FÜRTH, though the constant C_4 is not the same. But, as these formulae are used only for comparative measurements, this is of no significance.

As a general formula for the torsional moment, we use in practice (cf. equations 25, 26 and 30)

$$F = C \cdot \varepsilon \cdot V^2 \quad (31)$$

CHAPTER V

Sources of error in the ellipsoid method

The first studies with the ellipsoid method were made by FÜRTH (1924). It was soon found, however, that the execution of this method was marred by certain errors which tended to vitiate the results. In course of time additional sources of error, for which allowance had to be made, manifested themselves (see especially ORTHMANN, 1931, SHUTT, 1934, FISCHER and SCHIAFFELD, 1936).

Certain investigators who viewed the ellipsoid method with misgivings have alleged that its results do not tally with those obtained with other methods. These discrepancies, however, have subsequently been adjusted by investigations with improved apparatus (as regards glycine and urea, see DUNNING and SHUTT, 1938).

As methodical errors in DC determinations often elude observation, special attention should be given to any possibility of such errors. In the following, certain sources of error in the method, as well as special difficulties in connection with its use within the high frequency range, will be briefly discussed.

A. Galvanic polarization.

In the experiments made by FÜRTH, he used nickel as a material for the ellipsoid and electrodes. Nickel, however, is very liable to entail galvanic polarization (JONES and CHRISTIAN, 1935) and, as he also used alternating current with a low number of cycles (50 c/s), the errors were of considerable magnitude. For some time, the question as to how to avoid

polarization dominated research with the ellipsoid method, and several expedients were tried.

FÜRTH's disciple PECHHOLD (1927), for example, introduced platinizing. He too, however, used alternating current with 50 c/s. For this reason, the validity of his results was questioned by ZAHN (1927), who showed that, with such procedure, polarization voltages were bound to occur. MILICKA and SLAMA (1931), however, on controlling Pechhold's results with his original apparatus, found, within the normal errors of measurements, no deviation when higher frequencies (from 277 c/s to 2 212 c/s) were applied.

ORTHMANN (1931) employed ellipsoids and electrodes of silver and examined only silver solutions, in which he presumed that the polarization was low, though he reckoned with the possibility that it might have a certain effect. In view of later studies of polarization, however, it seems as if ORTHMANN had underestimated its importance.

JONES and CHRISTIAN (1935), in studies on galvanic polarization, obtained results which cast a clear light on the problem now under discussion. They measured the resistance and capacity in an electrode vessel where the distance between the electrodes and the electrode material could be varied. Polarization was found to be a minor additional resistance, which could be determined by varying the distance between the electrodes and extrapolating to zero distance, where the resistance exactly corresponded to the polarization. They found that Warburg's rule $\Delta R \cdot f^1 = \text{constant}$ applied to examined solutions between 500 and 4 000 c/s. Some of their results are reproduced in the following Table 1.

The table strikingly shows how extremely unsuitable nickel is as an ellipsoid material. It will be seen that silver, even in relatively strong solutions of silver nitrate, entails a distinct polarization, which, therefore, may be presumed to have occurred in ORTHMANN's determinations. It should also be

¹ ΔR denotes the increased resistance produced by the polarization, and f the frequency in c/s.

Table 1.

Polarization resistance ΔR for different electrode materials and electrolyte solutions at different frequencies, according to Jones and Christian.

Electrode	Electrolyte Normality	Frequency cycles per second				
		500	1000	2000	3000	4000
Ag	0.1 AgNO ₃	21.10	14.57	10.22	8.36	7.39
Ag	0.01 AgNO ₃	23.5	19.0	11.9	9.7	8.4
Ag	0.02 KNO ₃	57.2	38.0	26.1	21.6	19.0
Ag	0.01 KI	16.9	10.6	7.4	6.2	5.8
Ni	0.01 Ni(NO ₃) ₂	491	326	220	184	163
Ni	0.01 KNO ₃	398	273	192	158	139
Pt (smooth)	0.015 KCl	63.6	40.2	27.6	23.8	21.5
Pt (smooth)	0.01 KCl	57.7	37.2	25.9	22.0	20.7
Pt (platinized)	0.01 KCl	1.29	0.84	0.67	0.57	0.47

noted that polarization manifests itself, even at rather high frequencies, if *smooth* ellipsoids are employed. LIN (1936), like FISCHER and SCHAFFELD (1936), dispensed with platinizing and, instead, increased the frequency. ONCLEY (1938), even in the use of the bridge method, made large corrections to allow for polarization.

In view of the sensitiveness of the ellipsoid method to galvanic polarization, due regard should be paid to the errors which this may involve, even at the higher frequencies. If, after platinizing, the metal is heated to a red glow, a raw surface, the so-called »gray platinizing» will be produced. WHETHAM (1900) points out that, whilst it conduces, in some measure, to prevent polarization, it has considerably less adsorptive power than platinum black.

As the use of platinum black (for the reasons stated in the

following section B) should be avoided, and as frequencies of 100 kc/s were to be used, I tried gray platinizing. As no dependence on the frequency could be observed, and as gray platinizing was found to be durable and entailed no noticeable drawbacks, I used it throughout at radio frequencies, though without making any special study of its efficacy.

B. Adsorption.

The use of platinum black, on the other hand, entails many drawbacks. That its conductivity ($10^4 \text{ ohm}^{-1}\text{cm}^{-1}$) is lower than that of pure platinum ($10^5 \text{ ohm}^{-1}\text{cm}^{-1}$) seems to be of small consequence, but its power of adsorption is a source of annoyance. In weak solutions of electrolytes this effect is very marked, with the result that the concentration in the solution shows considerable variations. LEDERER (published by LIN, 1936) found that quite different concentration curves might be obtained according to the order in which the different concentrations were examined. As regards markedly surface-active substances, such as the proteins, the adsorption is bound to be still more pronounced and may be expected to have a very disturbing effect. SHUTT (1934) examined egg albumin with the use of a well platinized ellipsoid. As egg albumin is liable to be denatured at the surface, we must reckon with the probability that the ellipsoid will be covered with a coating of that substance. This will tend to modify the electrical properties of the ellipsoid and thus to affect the results. SHUTT moreover used very low frequencies, 100—200 c/s. At higher frequencies, the recurrent error due to a coating of albumin or other surface-active substances should be reduced by a condenser effect. With the use of gray platinizing, the author has found the same water value for nucleotides before and after measurement. The conductivity of highly diluted solutions of potassium chloride (0.001—0.005 molarity) does not change when the solutions are kept in a vessel with gray platinized electrodes. This indicates that, at the higher frequencies, the adsorption does not entail any appreciable significant error.

C. The moving system.

In order that the formula (31) on p. 36 shall be strictly applicable, the shape of the ellipsoid must be perfectly true. In practice, of course, this is unattainable. At the beginning of this investigation, great trouble, however, was taken to obtain the best possible ellipsoid shape, and special tests were made to study the effect of marked deviations from the ideal form. In these tests, an indirect reading method was adopted. It is based on the principle of compensating the deflexion of the ellipsoid in the electric field by turning the point of attachment of the suspended thread. Instead of an ellipsoid, a dumb-bell-shaped body may then be employed (ORTHMANN, 1931; cf. also COHN, 1931). As the direct and indirect reading methods gave the same results, and as the deflexion was strictly proportional to the square of the voltage (see Ch. VIII, p. 64), author was forced to the conclusion that, in direct reading, the practically inevitable deviations from the ellipsoid shape are of no significance. The use of oblate ellipsoids with high eccentricity, as in the present study, may conduce to improve the results.

In view of the inevitable fluctuations in voltage, the direct reading method involves practical advantages, and has therefore been mostly employed.

The sensitive systems used in the ellipsoid method may be affected even by weak surfacetension forces. The disturbing effect may be reduced if the surface-tension has a minute point of application. SHUTT (1934) passed only the thin suspension thread through the surface of the liquid. The author, however, found it necessary to immerse the entire moving system in the liquid.

D. Heat convections and mechanical disturbances.

As the sensitivity of the moving system must be high in order to enable the very weak torques to be measured, transmission of heat by the current in the solution is liable to have

a disturbing effect. Owing to thermal disturbances, PECHHOLD (1927) was unable to take measurements at a higher electric conductivity than at most $2 \cdot 10^{-3}$ ohm $^{-1}$ cm $^{-1}$. In order to avoid errors due to convection currents and to extend the range of measurement to still higher conductivities, ORTHMANN (1931) introduced a ballistic method. He sent a current shock through the electrode vessel and then measured the maximum deflexion made by the ellipsoid owing to the impulse. The ballistic method, however, involves certain difficulties. ORTHMANN made comparisons with an »air electrometer» constructed similarly as the ellipsoid apparatus and with an equally ballistic sensitivity. He found that, for different voltages and different durations of the current shock, there was a strict proportionality between the impulse and the maximum deflexion. This, however, is only the case if the time during which the voltage is applied is short relatively to the time of the maximum deflexion, as in ORTHMANN's determinations (about 0.2 seconds for making the connection, and 4.5 seconds pending the maximum deflexion). LIN (1936), on the other hand, used a relatively long connecting time (about two-thirds of the time for the maximum deflexion), which is bound to entail a methodical error. For solutions of polyribodesose nucleotides, in view of their viscosity, the ballistic method is not applicable. Nor was it adopted for other nucleotides, as it was found possible to take measurements at conductivities of about $3 \cdot 10^{-3}$ ohm $^{-1}$ cm $^{-1}$, without ballistic readings. For strong inorganic electrolytes, however, it seems to be preferable.

According to the author's experience, the distance between the electrodes must be at least three times as long as the ellipsoid in order to avoid the disturbing effects of local convective. Moreover the field strengths applied were low (down to about 1 volt cm $^{-1}$, but as a rule 3—4 volt cm $^{-1}$).

An electromagnetic wave motion at an electrode surface gives rise to a mechanical wave (BJÖRNSTÅHL and SNELLMAN, 1937). As the ellipsoid is extremely sensitive to mechanical stresses and tends to adjust itself with its axis of rotation at right angles to the wave front, this might be expected to

entail the result that the deflexions would be unduly small. But, as shown by control measurements (see Ch. VIII), this is not actually the case, at any rate as regards glycine and urea. At the low field strengths adopted here, such a wave motion is doubtless of very small intensity and does not seem to be of any consequence in practice.

E. Voltage variations and distortion.

For lower frequencies, alternating current generators with 100—500 c/s, and for higher frequencies electron tube oscillators, have been employed. It is difficult to obtain an alternating voltage with entirely constant amplitudes, especially at high frequencies. Precision in DC determinations with force methods, however, is greatly dependent on the accuracy of the voltage measurement. From the equation

$$F = C \cdot \epsilon \cdot V^2, \quad (31)$$

we obtain

$$\frac{|\delta \epsilon|}{\epsilon} = 2 \frac{|\delta V|}{V} \quad (32)$$

An error of $1/1000$ of the voltage, which must be considered to be very low, gives for water (DC ≈ 80) an incidental error of about 0.16. The accuracy of the individual measurements will therefore be greatly affected by the error in voltage measurement. As the deflexion of the ellipsoid is damped, rapid changes (within a few tenths of a second) would be of minor significance. Voltage measurements should nevertheless be made with a comparatively slow instrument.

In the use of high-frequency alternating current, several other difficulties are encountered. With high-frequency generators a satisfactory sine wave in the alternating current, especially at high effects, is not easily obtained. Deviation from the sine-wave form is explained by the occurrence of har-

monies. Owing to this so-called »distortion», we obtain, instead of

$$V = V_0 \cdot \sin \omega t,$$

the following relation:

$$V = V_1 \cdot \sin \omega t + V_2 \cdot \sin 2\omega t + V_3 \cdot \sin 3\omega t + \dots$$

where V_0 , V_1 , V_2 and so on are maximum voltages.

This distortion may have disturbing effects in several ways. Firstly, the inevitable losses through inductance and capacitance will become relatively greater as regards the higher frequencies, and secondly in the vicinity of an anomalous dispersion range, the higher frequencies may fall within another part of that range. The angle of deflexion, β , will then be

$$\beta = C (\varepsilon_1 \cdot V_1^2 \cdot \sin^2 \omega t + \varepsilon_2 \cdot V_2^2 \cdot \sin^2 2\omega t + \dots)$$

instead of

$$\beta = C \cdot \varepsilon (V_1^2 \cdot \sin^2 \omega t + V_2^2 \cdot \sin 2\omega t + \dots)$$

where ε_1 , ε_2 and so on are DC at different frequencies. Finally, the voltage measurement may be erroneous if a peak voltage meter is employed.

The effects of distortion, however, are of minor importance for low frequencies. ORTHMANN (1931) used two quite different forms of the ellipsoid method and compared the results when the one electrode vessel was filled with a control solution. Such a procedure serves, in marked degree, to compensate the variation in voltage and the distortion, but it is difficult to apply it at high frequencies.

In order to determine the voltage, the author tested different types of electron tube volt meters and thermocouples. As a thermocouple requires a series resistance by which the device would be made dependent on the frequency, an electron tube volt meter might seem to be preferable. This instrument, however, does not permit of an equally high degree of stability and precision. As the principal measuring instrument, I therefore used a thermocouple and, at frequencies over 2 Mc/s controll-

ed the dependence on the frequency with an electron tube volt meter. Between 100 kc/s and 2 Mc/s the electromotive force of the thermocouple closely followed the deflexion of the ellipsoid, whereas the electron tube volt meter showed some deviation. This — like the minor changes in the water value (see Ch. VIII) which were found at different frequencies —, was due to distortion, which, despite careful trimming, could not be avoided. Filters were tried, but it was found that a more practical procedure was to calibrate the apparatus for each frequency. At the highest constant frequencies it was easier to avoid distortion, and the difference in the water values could then be attributed solely to the dependence of the thermo-couple and its series resistance on the frequency.

F. Effect of the heterogeneity of the alternating field.

One of the essential conditions for the maintenance of a simple relation between the deflexion of the ellipsoid and the DC of the solution is that the metallic ellipsoid should be placed in a homogeneous electric field where the lines of force would otherwise have run parallel. In practice, however, it is difficult to produce such a field, as it requires relatively large electrodes in proportion to the distance between them. The author, like his predecessors, has taken a middle course, adjusting the size of the electrodes and the distance between them where it was uncertain whether the ellipsoid was in a homogeneous field. As the deflexion of the ellipsoid is also proportional to the square of the field strength, the effect of heterogeneity in the field would become still more marked.

For these reasons, a careful examination of the homogeneity of the alternating field seemed to be required. With the use of circular electrodes 6 cm in diameter and with a distance of 2.5 cm between them (*i. e.* the same dimensions and distance as in SHUTT's experiments), the same deflexion was found even where the ellipsoid had been shifted up to $\frac{1}{2}$ cm laterally or vertically. On the other hand, displacement of either electrode

towards the other would have more serious consequences, whence a deviation of the ellipsoid from the midway position by more than a few millimetres could not be permitted. In this central position no appreciable effect of the heterogeneity of the field could be observed. Nor was this the case with 5 cm electrodes, which accordingly were also employed.

In the use of radio frequencies, inevitable leakages occur to water baths, adjacent metallic objects, etc. These leakages are apt to be different for the leads to the two electrodes, and may vary in a way which seems to elude control. In the previous models of the apparatus used by the author, largish metallic objects in the vicinity were avoided, and the leads to the electrode vessel were made as short as possible. These precautions proved to be somewhat exaggerated and of minor importance. However, in order, as far as possible, to prevent the effect of such leakages in the field between the electrodes, the apparatus should be built as symmetrically as possible.

In order to obtain constant voltages at very high frequencies, it is essential that the outer circuit at some place should be securely earthed. LIN (1936), who used the ellipsoid method at a fixed frequency of 250 kc/s, had the one electrode earthed. In that case, however, the field strength in the electrode vessel, owing to leakages to the environment, will diminish according to the distance to the earthed electrode. As the leakages are of minor importance where the conductivity of the testing solution is high, the method will be to some extent dependent on the conductivity. For this reason, the potential of the ellipsoid should, instead, be kept as near earth as possible.

The apparatus used by the author, however, has proved to be so symmetrically built that, at frequencies up to about 1 Mc/s, symmetrical earthing, though it was actually used, would have been unnecessary. At still higher frequencies, however, the difficulties rapidly increased, and it was by no means easy to keep the electrode potential to earth constant and equal. Even with the aid of symmetrical earthing of the output transformer of the oscillator as well as of the electrodes, it was

not possible at 5.8 Mc/s and 12 Mc/s to obtain a secure and effective symmetrical earthing. A combination of symmetrical earthing and variable capacitive charging of the one lead therefore had to be resorted to. Also here, however, the earthing was found to consume such a large effect that the possibilities of measurement in electrically conductive solutions were limited.

CHAPTER VI

The Apparatus

During the course of the work the author's apparatus was several times rebuilt, in connection with attempts to extend the range of measurement to higher frequencies and to increase its precision. The first measurements were made at low frequencies (2 kc/s, 4 kc/s and 6 kc/s), but chiefly on standard substances. The preliminary results being encouraging, the possibilities of proceeding to the use of high frequency were examined. The difficulties then encountered related mainly to the high-frequency source, which, as regards a force method, must satisfy exacting requirements. An ideal oscillator should produce a large effect at a low output impedance, and yet give an output voltage with small distortion. And the frequency should be continuously variable within an extensive range. Moreover, in order to facilitate measurement, the frequency and the voltage should be practically free from fluctuations and independent of the load.

The first serviceable model of the apparatus was built for frequencies between approximately 100 kc/s and 1 Mc/s. It was found that the unavoidable fluctuations in the output voltage could be neutralized by a special technique. A number of determinations were made with it on polynucleotides and showed that it was very desirable to have facilities for the use of still higher frequencies. But, when the continuously variable frequency range was extended to 2 Mc/s, great difficulties were encountered owing to high-frequency losses. In order to proceed to still higher frequencies, it seemed evident that the further use of continuously variable frequency would have to be abandoned. A minor oscillator with three fixed frequencies (2.9 Mc/s, 5.8 Mc/s and 12 Mc/s) was accordingly built for this special

purpose. The difficulties which arose as the frequency increased were, however, greater than had been foreseen.

As the different models had given, within normal errors of measurement, practically the same results in standard solutions, I shall confine myself here to a description of the latest model of the apparatus. It consists of (1) an electrode vessel, with electrodes between which a moving system — the ellipsoid —, is suspended, (2) high-frequency sources (oscillators) and (3) instruments for the measurement of voltage and frequency.

A. General arrangement of the apparatus.

The mechanically sensitive parts of the measuring apparatus are mounted on a cement pillar (see Fig. 4). It is 1.5 m in

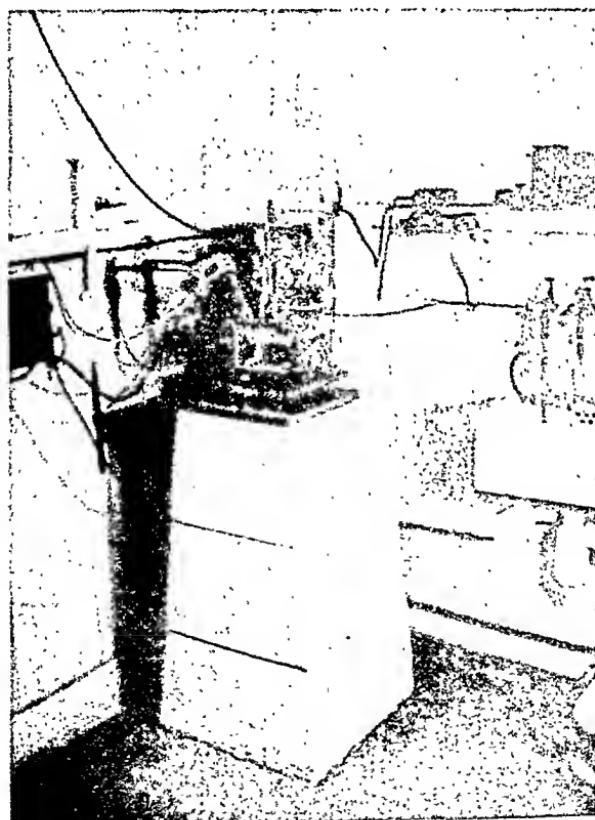


Fig. 4. General view of the arrangement of the apparatus in connection with the moving system.

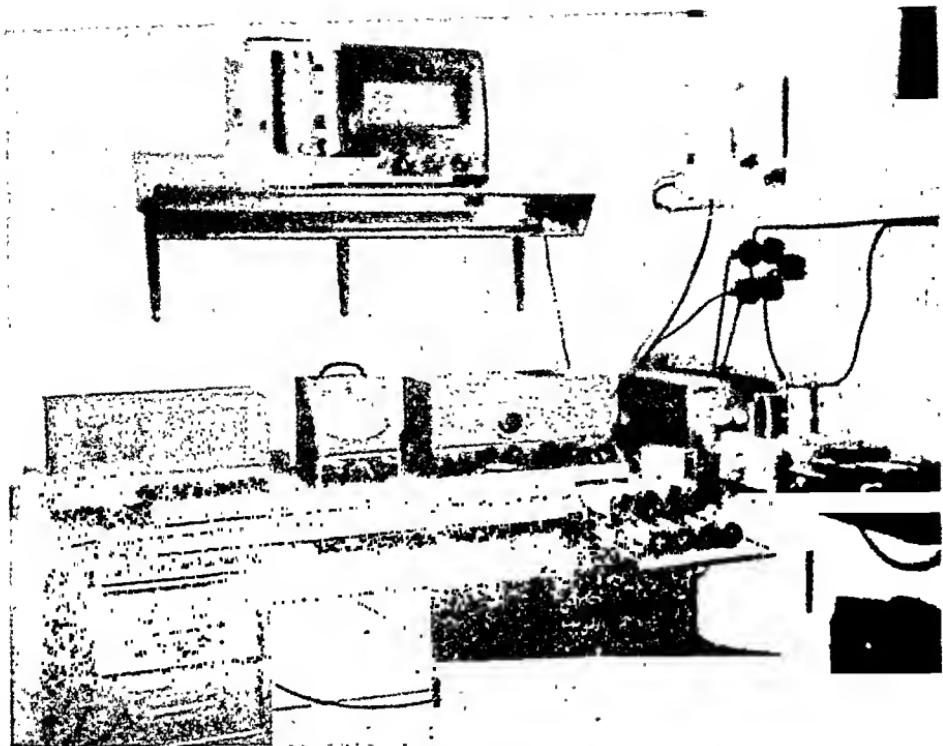


Fig. 5. *Reading device and oscillators.*

On the table we see from the left, the rectifier for the larger oscillator, the electron tube volt meter, and the variable oscillator. In front of the instruments the reading scale, one metre in length, is placed. On the shelf we see from the left the rectifier of the short-wave oscillator and the short-wave oscillator itself (with hood removed). Under the shelf to the right a voltage stabilization unit is placed. On the shelf above the table we see a wireless receiving set for frequency measurement.

height and has a ledge for the water bath and the electrode vessel at a level of 0.9 m. The pillar is made in three sections and stands on a cement base firmly embedded in the foundations. Between the sections lie sheets of pressed cork, 1 cm in thickness. Though the mechanical stability is quite satisfactory, slight disturbances are sometimes indicated by the drifting of the zero position.

During the measurements, the apparatus can be completely operated from the other part of the room, where a scale for reading the deflection of the ellipsoid, oscillators and various instruments are placed (see Fig. 5). This arrangement makes the apparatus more accessible and easier to operate than in

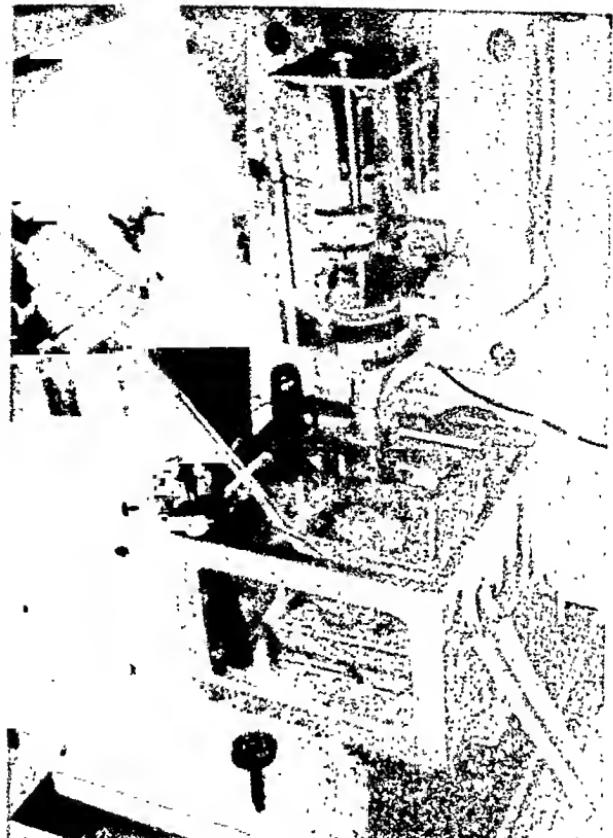


Fig. 6. View of the ellipsoid suspension system and the placing of the electrode vessel.

In the water bath we see the electrode vessel, which is fixed to the vertically movable cylinder. On the lid of the electrode vessel the thermocouple is observable to the left. Furthest to the left the motor of the stirrer and the illuminating lamp are discerned.

the previous models, where it had been reduced to a smaller compass in order to obtain short leads.

For the prevention of marked temperature variations which might affect the precision of the measuring instruments, there is a regulating device which keeps the room temperature at $20^{\circ} \text{ C.} \pm 0.5^{\circ}$.

B. Suspension device for the moving system.

Above the water bath there is a precision screw with a vertical axis, by which the moving system is suspended (see Fig. 6). The screw is of very stable construction and so de-

signed that the vertically placed axis can be turned by a worm with a horizontal axis. Wheels graduated to a scale of 1/100 and 1/10 000 rev., respectively, are fitted on the axes. The vertical axis is threaded at the top with a low pitch and, when detached from the worm, can be screwed up and down, which greatly facilitates replacement of the electrode vessel, the suspension of a new moving system, etc.

C. The moving system.

Apart from some preliminary attempts, oblate ellipsoids of platinum have always been employed. Different methods for the production of the ellipsoids were tested. It was found that the simplest procedure which could be adopted, without sacrificing precision, was to turn them in a lathe. A circular platinum plate is cemented on to a round brass rod of the same diameter. The rod is turned in the lathe and, with fine files and emery cloth, the edge of the platinum sheet is rounded off, to the pattern of a drawn ellipse. When the shape approximately tallies, the platinum sheet is reversed, carefully centred and ground on the other side. As a rule a good ellipsoid is obtained after reversing the platinum sheet three or four times. After platinizing, the ellipsoid is heated to a faint red glow, and then assumes a dull gray metallic hue.

The short axis of the ellipsoid was 0.1 or 0.2 mm in length and, for testing purposes, the length of the long axis was varied between 4 and 10 mm. As a rule, however, the length of the long axis was 6—7 mm and the axis ratio 1/60—1/70.

To the ellipsoid is fused a thin-walled glass capillary tube (G_1 in Fig. 7) about 3 cm in length, to the other end of which the suspension thread is fused. A mirror (M), 2 \times 3 mm in size, is attached with picein to the upper end of the capillary, at right angles to the short axis of the ellipsoid. The suspension thread (W') is fused to another glass tube (G_2), fixed to the vertical axis of the suspension screw and well centred.

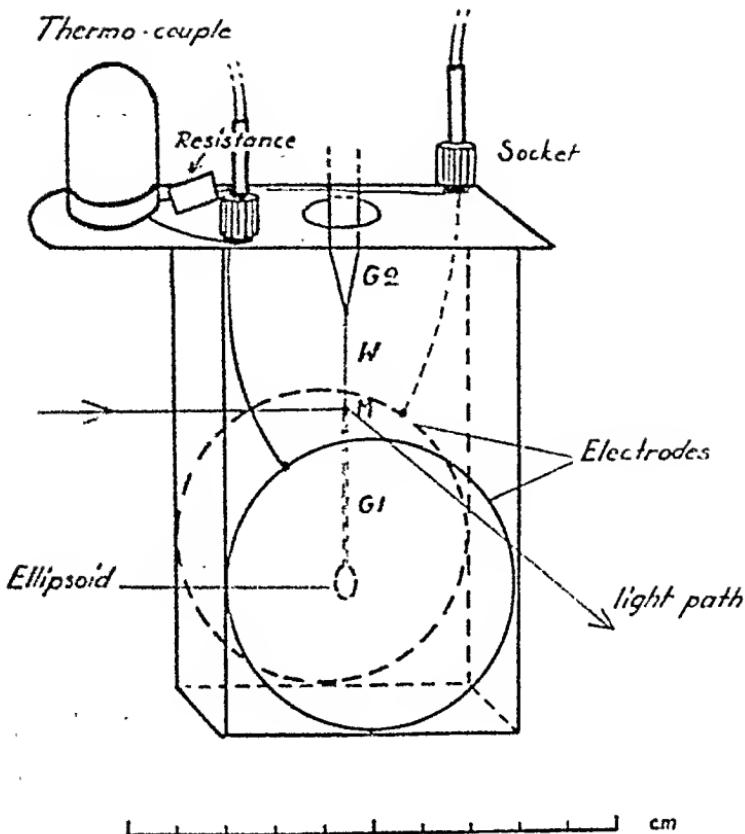


Fig. 7. Outline view of the moving system and an electrode vessel.

G_1 is a thin-walled glass capillary tube fused to the ellipsoid and to the suspension thread. G_2 is a glass tube fixed to the vertical axis of the precision screw. M denotes the mirror and W the Wollaston wire.

For the suspension of the moving system, Wollaston wires, 0.005, 0.007 and 0.01 mm in thickness, were exclusively employed. Experience had shown that the weight of the system and the dimensions of the suspension thread must be adjusted to the damping (viscosity of the testing solution). Thus, for the determination of polyribodesose nucleotides, a wire about 3 cm in length and 0.01 mm in thickness was usually employed. In the investigation of polyribose nucleotides, on the other hand, a relatively short wire (1—1½ cm in length and 0.007 mm in thickness) was found to be preferable.

The moving system was as a rule 60—70 mg in weight.

D. The electrode vessel.

Electrode vessels of glass and plexiglass, as a rule of rectangular shape and with a distance of 2.5 or 3 cm between the electrodes, were employed (see Figs. 6 och 7). The electrodes consisted of platinum sheets, 0.1 and 0.2 mm in thickness, or of 0.05 mm platinum foils. They were of circular shape, with a diameter of 5 to 6 cm. As a rule they were gray platinized, but in some cases smooth electrodes were used, without entailing any noticeable drawbacks. Different kinds of cement were used for fixing the electrodes. In a few cases the author attempted to make the contact surface between cement and solution as small as possible. So far as could be observed, the cements, which as a rule were picein or rubber mastic, did not entail any inconveniences. A volume of 120—250 ml testing solution was needed.

For the connection of the electrodes to the oscillator, the author uses a platinum wire, 1 mm in thickness, spot-welded at the electrodes and running along the inner wall of the electrode vessel to sockets on its lid. There it is connected with the wires from the voltage measuring instruments and from the alternating current generator. The lid of the electrode vessel on one of the short sides was extended, to provide room for the thermocouple and its multiplier. This arrangement conduces to shorten the wires to those instruments (about 3 and 8 cm, respectively).

The electrode vessel stands in a water bath of glass with a metal framing, holding about 6 litres. To the suspension screw is attached a cylinder movable about the lower part of the vertical axis of the screw. By screwing the cylinder against the lid of the electrode vessel, satisfactory stability is obtained, and stirring in the water bath can proceed even during measurement. The temperature in the water bath is maintained at 20.0° C. $\pm 0.05^{\circ}$.

E. Reading device.

The deflexion of the ellipsoid is measured optically. As indicated by Fig. 7, a pencil of rays enters through the short side of the electrode vessel towards the mirror of the moving system, is reflected at right angles and is thrown by another mirror on a reading scale (see Fig. 5). In front of the source of light there is a wire, 0.1 mm in thickness, which is projected on the scale. The light path between the mirror of the moving system and the scale is 3.6 metres in length. The use of such a long light path is not intended to increase the precision of measurement, but is due to the necessity of adopting a special measuring technique in view of the unavoidable fluctuations in voltage (see Chapter VII). The scale is graduated in millimetres and provided with two sliders.

F. High frequency sources.

As previously mentioned, two oscillators were used, namely a larger high-frequency oscillator for intermediate and long wave-lengths and a minor oscillator for the short-wave range. The latter, however, was used only for the completion of dispersion curves.

The larger oscillator for frequencies between 100 kc/s and 2 Mc/s, as indicated by the circuit diagram (Fig. 8), consists of a oscillator stage, followed by an amplifier stage in a push-pull coupling. For both stages, the anode load consists of a primary tuned transformer. As a power stage lies between the oscillator tube and the output circuit, changes in load resistance, practically speaking, do not affect the frequency.

The generator is intended both for low and high frequencies. Within the former range the frequency can be adjusted to three fixed positions (2 kc/s, 4 kc/s and 6 kc/s). The high frequency is continuously variable between the limits of about 100 kc/s and 2 Mc/s, corresponding to the wave-length range of 150—3 000 m. Fig. 8 shows the connection of the generator

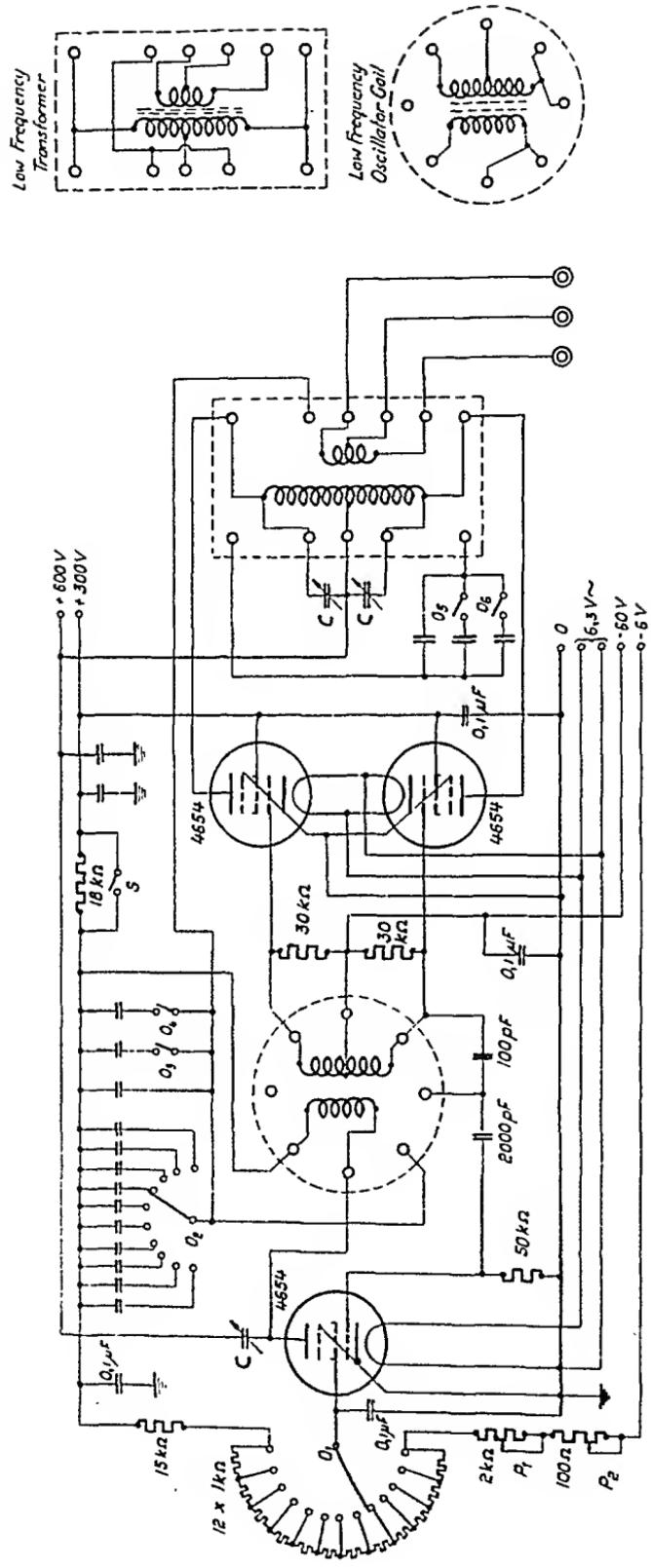


Fig. 8. Wiring diagram for oscillator with low and high frequency.

Frequency ranges: low frequency 2 kc/s, 4 kc/s and 6 kc/s, high frequency variable between 100 kc/s and 2Mc Output impedance: 15, 30 and 80 ohms. Power output: about 50 watts. O is a stage switch for rough adjustment and P_1 and P_2 are potentiometers for fine adjustment.

at high frequency. The required changing-over between the low and high frequency ranges takes place automatically on the exchange of coils.

There are three outputs for different load impedances, *viz.* for 15, 30 and 80 ohms. The output voltage can be continuously regulated from zero to 30—40 volts by adjusting the screen grid voltage of the oscillator tube. In order that this regulation shall be as stable and precise as possible, a stage switch (O in Fig. 7) is used for rough adjustment and two potentiometers (P_1 and P_2) for fine adjustment. The maximum power output is about 50 watts.

The frequency range from 100 kc/s to 2 Mc/s is covered by 5 sets of coils. Tuning within each range is effected by the condenser C , which has three sections. These sections are connected up in such a way as to ensure simultaneous tuning of the oscillator circuit and the symmetric output circuit.

The wave shape, as can be observed on a cathode ray oscillograph, is perfectly sinefoidal within the entire frequency range.

The oscillator is driven from a rectifier unit connected to 220 volt alternating current. The alternating voltages are carefully filtered in order to avoid a 100-period hum voltage.

The fluctuations in voltage from the mains were at times very marked and then conducted to prevent measurement. A voltage stabilization unit (Siemens) was therefore connected before the rectifier. It has an output voltage of 220 volt $\pm \pm 1\%$ for voltage fluctuations of at most $\pm 15\%$.

The short-wave oscillator for fixed frequencies (2.9 Mc/s, 5.8 Mc/s and 12.0 Mc/s), as indicated by the circuit diagram (Fig. 8), consists of an oscillator circuit with a tube, in whose anode circuit the second overtone is obtained, and a power circuit. Thanks to the device of a buffer stage between the output circuit and the oscillator circuit, a moderate load has a very slight effect. The output voltage is shortcircuited by a variable condenser. In the case of marked external resist-

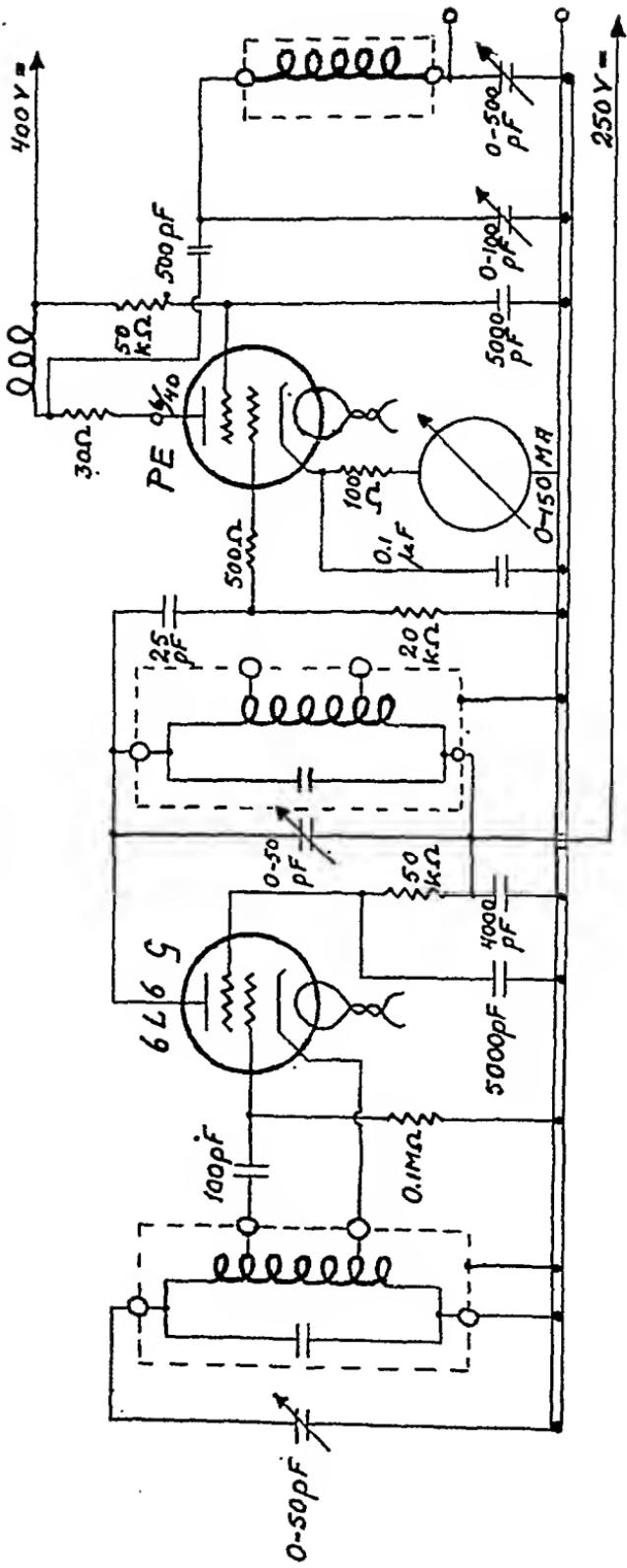


Fig. 9. Wiring diagram for short-wave oscillator.

Frequencies 2.9 Mc/s, 5.8 Mc/s and 12 Mc/s.

ances the capacity must be increased by detached condensers, as otherwise the output voltage would be too high. The degree of distortion is very low, being less than 0.5 % at an output effect of at most 12 watts. The oscillator is fed through a rectifier unit (intended for short-wave transmitters, AGA type R 3—38347), connected to the mains. The short-wave oscillator has the disadvantages of a relatively high output impedance, especially at high load, and an unsymmetrical output.

The wiring between the oscillators and the electrode vessel consists of two intertwined leads, each consisting of 28 wires, over 5 metres in length. As regards the larger oscillator, the losses are insignificant, and even for 12 Mc/s (the highest frequency used), the voltage drop in the wiring, at the highest loads used, is less than 20 per cent.

This loss of effect may *per se* seem rather considerable; but even with the use of short wiring the serviceableness of the minor oscillator in conductive solutions is greatly restricted.

Symmetrical earthing was adopted in two forms. Firstly the output transformer for the larger oscillator was short-circuited via a potentiometer (40 ohms, 100 watts) with an earthed contact arm; and secondly the electrodes were earthed over a 200 ohms carbon resistance (2 watts). As the apparatus had been built with a view to the greatest possible symmetry, symmetrical earthing was not required at frequencies of less than 1 Mc/s. But, as symmetrical earthing was necessary at higher frequencies, it was used also at the other frequencies.

As regards the short-wave oscillator, the electrodes were earthed at 500 ohms and a variable condenser ($500 \mu\mu F$) was connected between the one electrode and earth. By varying the capacity, the same voltage drop between each of the electrodes and earth was obtained. At higher conductivities in the testing solution, however, the voltage drop was not the same on repeated connections of the voltage, whence the values were not reproducible.

G. Frequency determination.

The frequency was determined with the aid of a wireless receiving set (Centrum, type KW 6), connected to the mains. The receiver was calibrated with a »Heterodyne Frequency Meter» (General Radio Co., type 615) and was checked against broadcasting stations.

H. Voltage measuring.

The voltage over the electrodes was measured with a thermo-couple, connected via a carbon resistance, to as short wiring as possible. The thermo-couple (Philips, type Th 2) has a straight resistance wire, to which the thermo-element is directly soldered. The thermo-couple gives 12 mV e.m.f. for 10 mA, but, as the electromotive force was exactly proportional to the square of the current strength, not more than 5 mA were actually used. The carbon resistances (500—3 000 ohms, Vitrohm) will stand a load capacity of two watts. They are placed close to the thermo-couple, whose electromotive force is measured by a Cambridge Pot Galvanometer with an internal resistance of 1 000 ohms and a stated sensitivity of 170 mm/ μ A at a distance of one metre. In order to obtain a sufficient deflexion on the galvanometer without the use of unduly strong current through the thermo-couple, it is not always possible completely to adjust the external resistance of the galvanometer to the internal resistance. Even a rather marked damping of the galvanometer was found to be of no significance, as the reproducibility (tested with storage-battery voltages) was nevertheless satisfactory, and as the slow action of the thermo-couple in that respect was the determining factor. For the control and estimation of the high-frequency losses, an electron tube volt meter (Philips GM 4150) was employed. It has an input capacity of about 10 $\mu\mu$ F, which gives too low an impedance to permit neglect of the effect of the instrument at the highest frequencies.

CHAPTER VII

Measuring technique and precision

In determining DC with the ellipsoid method, the torsional moment for the ellipsoid in the testing solution is measured and compared with the torsional moment for the same voltage in a solution with a known DC. Throughout this investigation the reference solution was distilled water ($DC = 80.3$ at $20^\circ C$), which was examined in immediate connection with the measurement of the sample.

In taking measurements, three different readings should, strictly speaking, be made at the same time, *viz.* for (1) frequency, (2) voltage and (3) the deflexion of the ellipsoid. But, as this would be very difficult to carry out in practice, the high-frequency oscillators, as previously mentioned (Ch. VI), were built with a view to such stability that fluctuations in frequency could be ignored. At different output voltages and loads, the frequency was in fact found to be so constant that, after adjustment before measurement, it could be maintained practically unchanged (variation $< \frac{1}{2} \%$).

The voltage, on the other hand, did not keep quite constant, especially at high frequency. A variation in voltage of 0.5 per cent. gives an error in DC of 1 per cent. In order to reduce the error due to variations in voltage, technical arrangements were made to ensure that the deflexions of the ellipsoid and of the galvanometer which measures the electromotive force of the thermocouple should go in the same direction and could be followed on the same scale. The series resistance of the galvanometer and the thermocouple were adjusted so that the deflexion of the galvanometer was approximately double that of the ellipsoid deflexion, and so that the galvanometer and

ellipsoid were equally slow in action. The galvanometer was then adjusted on the zero position of the scale, and the ellipsoid at half the expected deflexion for the voltage. The two deflexions will then lie close to one another on the scale and can conveniently be read at the same time.

The filling of the electrode vessel had to proceed very cautiously in order to avoid exposing the Wollaston wire to excessive mechanical strain, which would manifest itself by a change in the zero position of the ellipsoid and its consequent instability.

So far as could be observed, the deflexion of the ellipsoid was not affected by changes in the level of the liquid in the electrode vessel, presumably because the free surface of the liquid was always rather high above the electrodes. However, as the electrode vessel was always filled in such a way that the point of attachment of the suspension thread just dipped into the water, the level of the liquid was approximately constant for each moving system.

The oscillators and electron tube volt meter must be connected at least half an hour before measurement. During some of the longer measuring periods they were kept connected without interruption.

The measurement procedure is as follows. When the testing solution has reached constant temperature, the oscillator is adjusted at the required wave-length with the aid of the radio receiver. With an electron tube volt meter, the potential of the electrodes is determined relatively to earth. If it is not the same, it is equalized by varying the capacity of a condenser connected from one of the electrodes to earth. The one slider of the reading scale is adjusted to the zero position of the ellipsoid. The voltage is applied to the electrodes, whilst the other slider follows the deflexion of the ellipsoid and is adjusted to the mirror image when the voltage has been maintained constant for a few seconds. The galvanometer deflexion is read and the voltage is disconnected. If the zero position of the ellipsoid has shifted somewhat, the mean

value of the zero position before and after measurement can be used. The deflexion was in fact found to be additive to the weak forces which cause the drifting of the zero position.

A measurement, as a rule, takes 10—20 seconds, but between the measurements the ellipsoid does not resume a stable position until after the lapse of up to 10—15 minutes, especially when examining electrically conductive solutions. Unless mechanical or electric disturbances had arisen and prevented further measurement, at least ten readings were made.

The determination of DC in a solution at one and the same frequency (including the control of water value, tempering of the solution etc.) usually takes 4—5 hours. In the short-wave range, where the testing solution and water has to be examined alternately several times, it often happens that not more than one measuring point per day can be determined.

For each reading, the quotient ellipsoid deflexion/galvanometer deflexion was computed. This quotient is proportional to DC and was taken as a basis for the computation of the mean value and the standard deviation, whereupon DC was computed from it. The mean error, Q, of the arithmetical

medium is calculated from the formula $Q = \sqrt{\frac{\sum \Delta^2}{n(n-1)}}$,

where Δ = the deviation from the mean value and n = the number of observations. In general, the mean error of the medium was ± 0.2 DC units.

As regards the reproducibility from day to day, the measurements distinctly showed that it is greater for certain substances than for others. The above-mentioned quotient is rather constant *e.g.* for water ($= 80.3 \pm 0.15$) as well as for polyribodesose nucleotides and glycine, whereas polyribose nucleotides, potassium chloride and also urea show greater differences at different times of measurement (maximum difference 1 DC unit). The accuracy is determined in the first place by the electric conductivity, secondly by the frequency used, and finally by certain unknown factors, which must be attributed to the testing solutions.

CHAPTER VIII

Control of the method

Dielectric investigations have in many cases shown bad correspondence. The disparities are not only due to the use of different methods: they have often occurred in different executions of the same method. In regard to solutions in which the electric conductivity has not been very low, the discrepancies have been particularly marked. As regards non-conductive solutions, thanks to careful determinations, a number of substances which are suitable for testing purposes have been investigated. For electrically conductive solutions, on the other hand, this is not the case. In these circumstances, a control of the technique must be confined to (1) testing of details in the apparatus where errors are expected to occur, (2) control determinations of solutions of non-conductive test substances and (3) examination of electrolytic solutions in order to ascertain whether the results are probable.

Some typical examples of procedures for the control of the method will now be described.

A. Continuous control.

A thermocouple does not give complete proportionality between the electromotive force and the square of the current strength. The thermocouple used in this investigation (Philips Th 2) should show a deviation of less than 2 % for an electromotive force of 12 mV. On control with direct current and alternating current of 50 c/s, a strict proportionality between the electromotive force and the square of the current strength has been found for current strengths of up to about 5 mA. In such cases the precision of the thermocouple and of the galva-

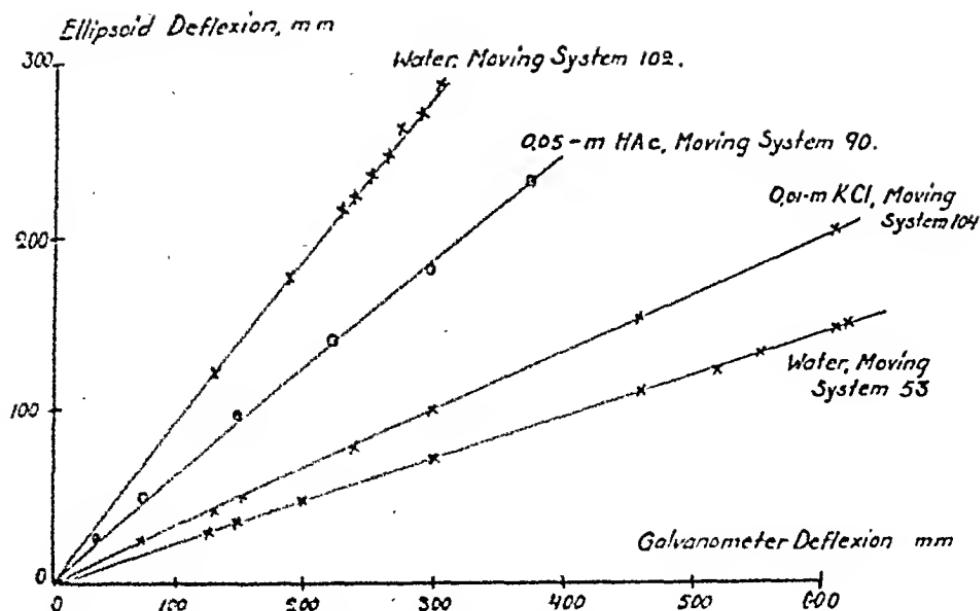


Fig. 10. Examples of measuring series with continuous control of the method.

nometer employed is so high that, on testing with storage-battery voltages, exactly the same values — within normal errors of reading — are obtained.

As indicated by the formula (26), the deflexion of the ellipsoid should be proportional to the square of the voltage, i. e. in the present case the deflexion of the galvanometer. It is important to control this, as methodical errors due to polarization, local convections and distortion of the measuring voltage etc., will result in deviations.

With a view to continuous control of the method, the proportionality between the deflexion of the galvanometer and that of the ellipsoid was ascertained for each series of measurements. Examples of such series are given in Fig. 10, in which, besides water, some values of potassium chloride (KCl) and acetic acid (HAc), for different moving systems, have been included.

Continuous control diminishes the risk of methodical errors and, when an unknown solution is being examined, gives one a certain assurance that the method was applicable to the solution in question.

B. Measurements at different frequencies.

At different frequencies somewhat varying water values are obtained, either because the series resistances to the thermo-couple, despite the testing of specific resistances, are not completely independent of the frequency, or owing to the occurrence of a low, but disturbing, degree of distortion.

The testing of standard substances for control purposes therefore comprised determinations of glycine and urea solutions at different frequencies. It was found that, within normal errors of measurements, the same increment was always obtained, even at wave-lengths where the water values were different.

An example of such a test is given in Table 2 and Fig. 11. It was made on a 3.5 molar urea solution, with moving system No. 112. The system was relatively non-sensitive, but was

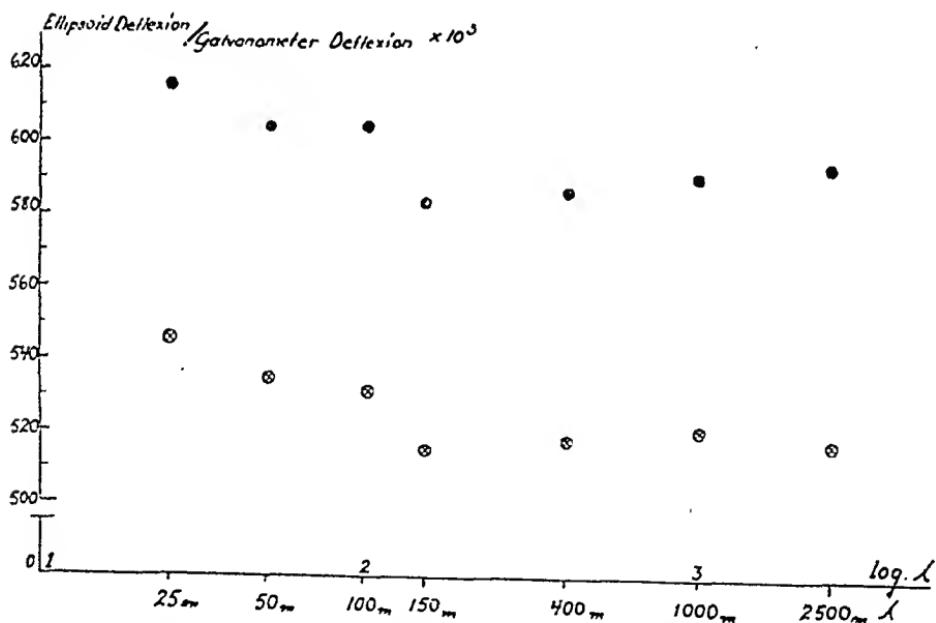


Fig. 11. The quotient between ellipsoid and galvanometer deflexion for water and urea (3.5 molar solution) at different frequencies.

⊗ water values
● urea values

Table 2.

*The quotient between ellipsoid and galvanometer deflexion for water and urea (3.5 molar solution) at different frequencies.
Temp. 20° C.*

Wave-length m	Quotient for water	Quotient for urea solution	DC of the urea solution	Incidental error of DC	$\varepsilon - \varepsilon_0$
25	0.5455	0.6180	91.0	0.25	10.7
51.7	0.5345	0.6040	90.7	0.14	10.4
103.4	0.5812	0.6042	91.3	0.12	11.0
150	0.5152	0.5882	90.9	0.08	10.6
400	0.5180	0.5860	90.8	0.04	10.5
1000	0.5205	0.5900	91.0	0.10	10.7
2500	0.5178	0.5912	91.8	0.16	11.5

ε_0 denotes the DC of water ($= 80.3$) and ε denotes the DC of the urea solutions.

selected because it had been used in marking a number of points on the thymonucleic acid dispersion curve. The ellipsoid had the axis ratio $1/75$ and the system was suspended by 21 mm wire of the dimension 0.01 mm. Apart from the frequencies under 150 m, the water values lie within $\pm 0.5\%$ and vary within a DC unit. That the water and urea values lie higher at short wave-lengths is due to the fact that the high-frequency losses in the series resistances are larger there. As will be seen, the increment is fairly constant.

C. Control with solutions of glycine.

WYMAN and MC MEEKIN, with a resonance method at a wave-length of 2—5 m, found that glycine had a molar increment of 22.8 at 20° C. HEDESTRAND (1928), with the use of a bridge method and 900 kc/s, found the increment to be 23.0 at 20° C. Glycine has also been studied with the ellipsoid

method, for example by FÜRTH. As previously mentioned, however, his execution of the method was unsatisfactory and its results misleading. Later on, DUNNING and SHUTT (1938) at 1 000 c/s found a molar increment of 24.5. The results of the tests with the ellipsoid method thus showed good correspondence with those of other methods, and glycine could therefore be considered to be a suitable testing substance.

In the author's control tests, glycine acc. to SØRENSEN (Kahlbaum) was examined after double crystallization, as a rule only in a few concentrations. Some results for different concentrations are shown in Table 3 and Fig. 12.

As will be seen, my values closely correspond to those of DUNNING and SHUTT, and in the controls a somewhat higher value was obtained throughout than that found by WYMAN and MC MEEKIN.

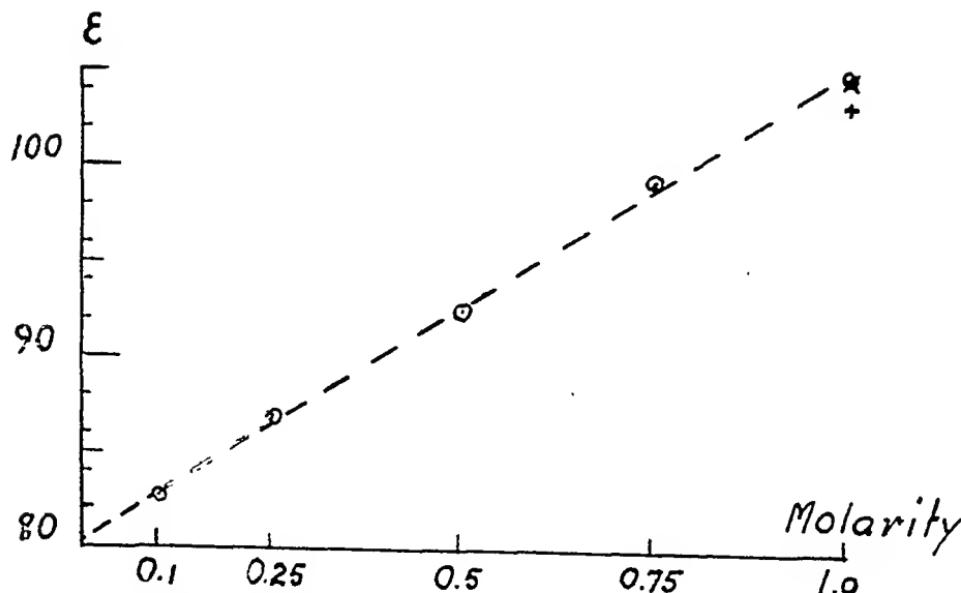


Fig. 12. DC of glycine at different concentrations.

- + Wyman and Mc Meekin's value.
- × Dunning and Shutt's value.
- Author's own values.

Table 3.

*DC of glycine at different concentrations. Temp. 20° C.
Wave-length 1200 m.*

Concentration <i>c</i> (molarity)	DC	Incidental error of DC	$\epsilon - \epsilon_0$	Increment $I = \frac{\epsilon - \epsilon_0}{c}$
0.1	82.8	0.17	2.5	25.0
0.25	86.9	0.28	6.6	26.4
0.5	92.5	0.28	12.2	24.4
0.75	99.2	0.10	18.9	25.2
1.0	104.9	0.24	24.6	24.6

ϵ_0 denotes the DC of water ($= 80.3$) and ϵ denotes the DC of the glycine solution.

D. Control with solutions of urea.

WALDEN and WERNER (1927) have shown that the increment of urea is liable to be affected by impurities, and that urea solutions after some time undergo changes in DC according to the concentration. After WYMAN's (1933) determinations, which are in fairly good correspondence with those of HARRINGTON (1916), KOCKEL (1925), WALDEN and WERNER (1927) and others, the DC for urea solutions may be considered to be ascertained and urea to be serviceable as a standard substance. With the ellipsoid method, DUNNING and SHUTT (1938) for a 1.0 molar solution at 20° C, found a ratio of 1.034 to water, which tallies well with WYMAN's figures.

Like glycine, urea was used by the author for control of the method. The above-mentioned observations of WALDEN and WERNER were on the whole confirmed. However, on examination of a several times crystallized preparation (Merck) within 4—5 hours, no change could be observed.

A concentration curve, including also WYMAN's values, is shown in Fig. 13. The values are reproduced also in Table 4.

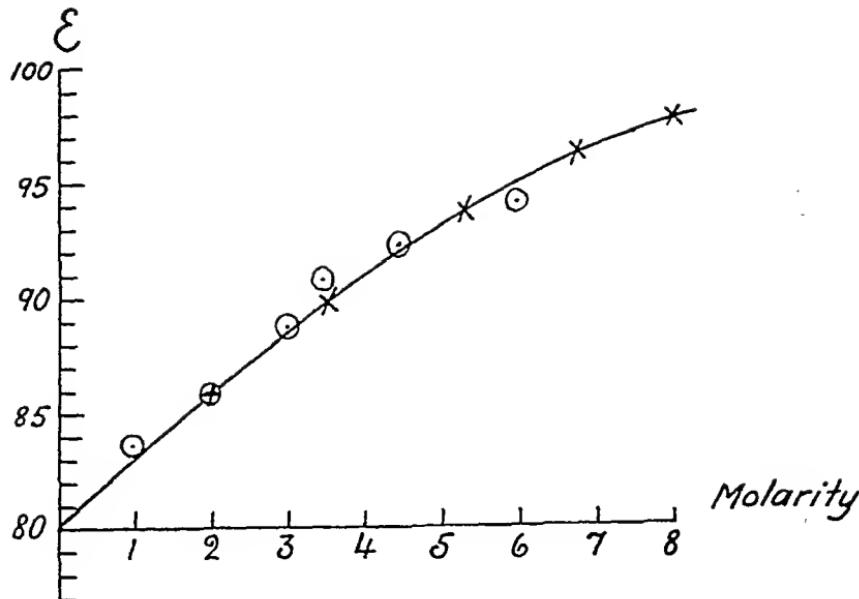


Fig. 13. DC of urea at different concentrations.

—×— Wyman's values.
○ Author's own values.

Table 4.

DC of urea at different concentrations. Temp. 20° C.
Wave-length 1200 m.

Concentration <i>c</i> (molarity)	DC	Incidental error of DC	Increment $I = \frac{\varepsilon - \varepsilon_0}{c}$	Increment $I = \frac{\varepsilon - \varepsilon_0}{c}$ after Wyman ¹
1.0	83.4	0.16	3.1	2.80
2.0	85.8	0.17	2.8	2.75
3.0	88.6	0.19	2.8	2.68
3.5	90.7	0.24	3.0	2.67
4.5	91.9	0.23	2.6	2.58
6.0	94.1	0.10	2.8	2.45

¹ interpolated values.

ε_0 denotes the DC of water ($= 80.3$) and ε denotes the DC of the urea solution.

The correspondence is on the whole quite satisfactory. Evidently, however, it is much more difficult in urea to obtain such a high degree of reproducibility as *e. g.* in glycine.

E. Control with solutions of potassium chloride.

As regards the strong electrolytes, the variations in DC have not been definitely ascertained. According to DEBYE-FALKENHAGEN's theory, owing to the deformation of the ionic atmosphere in the electric field, an asymmetry of the charge is formed. The polarization, and thus DC, will then increase. Thus, according to the theory, strong electrolytes give rise to an increase in DC (see FALKENHAGEN's monography: »Electrolytes», Oxford 1934).

The experimental results have been of a very shifting character (see the surveys by BLÜH 1926, 1934 and, as regards more recent investigations, GRUBB and HUNT 1939). It seems probable that DC at rising concentrations of potassium chloride first falls and then rises above DC of the water.

It is, however, quite permissible, for control of the method and its accuracy, to use an electrolyte solution even if we do not know its DC. No systematic investigation has been made, but preliminary determinations have shown an initial fall of about half a DC unit for 0.001 m potassium chloride. The DC then rises and, for about 0.003 m potassium chloride solution, within normal errors of measurement, gives the same value as water. A 0.003 m potassium chloride solution was accordingly used in order to control the method's independence of the conductivity, at different frequencies. When the symmetrical earthing had been made effective, this was found to be the case also at the highest frequencies used.

Summary, Part II

For a study of the dielectric properties of polynucleotides, the author has taken up the ellipsoid method indicated by FÜRTH. Chapter III records the points of view which determined the choice of method, namely (1) that it should permit an electric conductivity of about 10^{-3} ohm $^{-1}$ cm $^{-1}$ and (2) that measurements could be made at different frequencies within the radio frequency range. No method which satisfies these requirements had previously been available.

Chapter IV summarizes the relations given by FÜRTH and BJÖRNSTÅHL for the ellipsoid method. The deflective force of a rotation ellipsoid in an electric field — in addition to the dimensions of the ellipsoid —, is dependent on the DC of the solution, the field strength in the second potency and the angle of the axis of rotation to the field. The frequency and conductivity have no disturbing effect and, in comparative determinations with solutions of known DC, the deflexions at the same voltage are proportional to the DC of the solution.

The ellipsoid method has several sources of error, some of which, and the possibilities of their neutralization, are discussed in Chapter V.

In Chapter VI the author reports the practical execution of the method with the apparatus employed. For a continuously variable frequency between about 100 kc/s and 2 Mc/s, the apparatus in this investigation was used at $3 \cdot 10^{-3}$ ohm $^{-1}$ cm $^{-1}$, and gave accurate values at still higher conductivities. At a conductivity of $5 \cdot 10^{-4}$ ohm $^{-1}$ cm $^{-1}$ and under, the mean error of the medium is, as a rule, about $\pm 0.25\%$. For less conductive solutions (at most $2 \cdot 10^{-4}$ ohm $^{-1}$ cm $^{-1}$) the range of frequency could be extended to some fixed frequencies in the short-wave region, the highest being 12 Mc/s.

Chapter VII gives a description of the measuring technique, which aims especially at reducing the effect of inevitable variations in voltage.

Finally, in Chapter VIII the author gives a survey of the details of the apparatus which, in the use of the ellipsoid method, must be controlled, and adduces some examples of control determinations of urea and glycine. The results are in fairly good correspondence with those of other methods.

PART III
EXPERIMENTS

CHAPTER IX

Some data regarding the polynucleo- tides examined

A. Previous investigations.

The present work relates only to the dielectric properties of polynucleotides. I shall therefore confine myself here to reviewing some previous investigations of immediate interest for the interpretation of my results.

In the study of physiological substances, it is important to consider how far the preparations reflect the properties of the substances *in the organism itself*. In the preparation of polynucleotides, it is essential to avoid treatment with acids or alkalies, which is bound to entail changes in those substances.

The first method for the preparation of polynucleotides with a neutral reaction and at low temperature was that indicated by HAMMARSTEN (1924) for thymonucleic acid. Subsequently, with gentle means, also polyribose nucleotides were produced from different materials (HAMMARSTEN, not yet published).

HAMMARSTEN (1924) made a thorough study of thymonucleic acid, with physico-chemical and other methods. An aqueous solution of its neutral sodium salt had a very high viscosity, and he found that the increase in viscosity with rising concentration was greater for solutions containing more than about 0.2 g/100 ml (Fig. 14). This indicates aggregation at higher concentrations. The conductivity as well as the viscosity changed with time. With a concentration of sodium thymonucleinate of 0.156 g/100 ml, the viscosity fell by 10.5 % in 20 hours, whilst the conductivity rose by about 1 %. The viscosity was also found to be extremely dependent on small

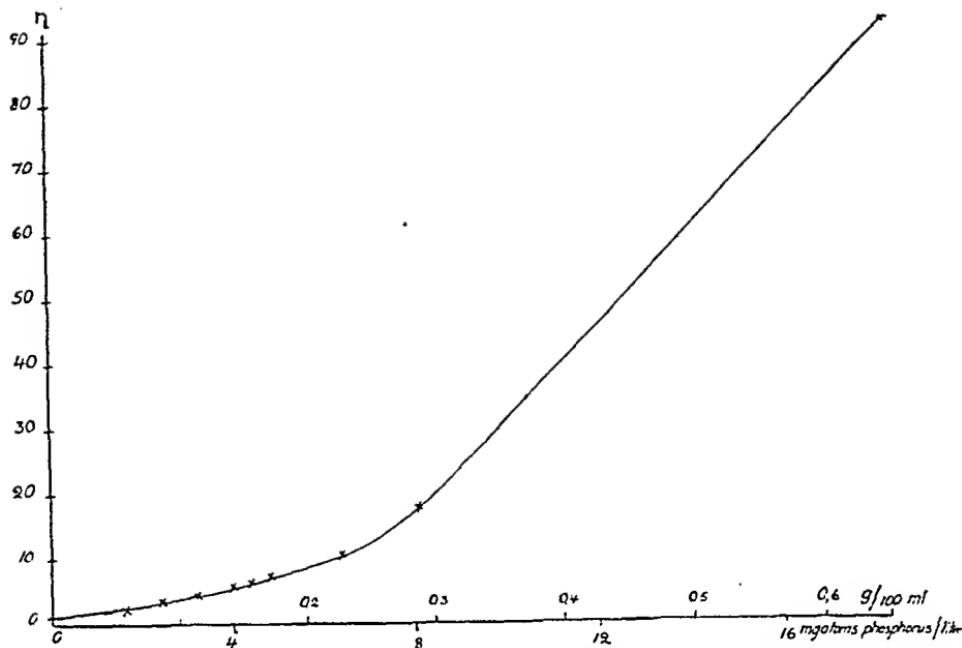


Fig. 14. Viscosity of neutral sodium thymonucleinate at different concentrations (after HAMMARSTEN, 1924).

additions of salt, and as regards a 0.2 solution it fell by 23 % if sodium chloride was added to a molarity of $0.5 \cdot 10^{-3}$.

In studying the osmotic pressure, HAMMARSTEN (1924) found that it was dependent on the size of the positive ion, so that salts with large positive ions showed a higher osmotic pressure, whereas small ions were osmotically less active (HAMMARSTEN's volume effect). For the free thymonucleic acid, the osmotic pressure was even less than could be computed from the hydrogen ion concentration. Proceeding from thermodynamic considerations, LINDERSTRØM-LANG (1926) explained the HAMMARSTEN effect by assuming that the activity of the thymonucleic acid is a diminishing function of the concentration. This, however, is the case only if a very marked interaction between the nucleic acid molecules is taking place. The positive ions would then exercise a screen effect, which would be less marked the larger the ion.

PEDERSEN (1940) studied HAMMARSTEN's thymonucleic acid preparation in an ultracentrifuge. The sedimentation rate

was found to correspond to a molecular weight of 200 000 in dilute solutions (0.035 %) and was still greater at higher concentrations. The diffusion constant (computed from the sedimentation curves) was four times greater than might be expected from the coefficient of friction, indicating that the particles were not exactly of the same size.

On the basis of the results from experiments on double refraction of flow (SIGNER, CASPERSSON and HAMMARSTEN, 1938), the molecular weight has been estimated at 500 000—1 000 000, with the axis ratio 1/300. As the molecules, in a marked degree, were negatively double refracting, it was supposed that the purin and pyrimidine rings lie perpendicular to the longitudinal axis, arranged in a definite pattern.

The regularity in the thymonucleic acid molecule that could be ascertained by a study of the double refraction of flow was observed also on examination with the X-ray. ASTBURY and BELL (1938) found a strongly-marked period of about 3.34 Å along the fibre axis of an extended, almost dry, preparation. They state that, though this spacing might seem unduly short for the distance between the mononucleotides, it corresponds with what might have been expected from the density (1.62—1.63 g/ml). It is of great interest to note that they observed also longer periods, corresponding to at least 17 mononucleotides.

Thymonucleic acid must thus be considered to have long, rod-shaped molecules with a very high molecular weight, even in dilute solutions. It shows a very regular structure, with a close succession of mononucleotides, but the occurrence of also larger, regularly recurring units is considered probable.

As regards the polyribose nucleotides, physico-chemical investigations are very scarce. Difficulties in preparation have evidently stood in the way, especially in view of the risk of enzymic decomposition.

In low-polymeric preparations, determinations of molecular weight are difficult and uncertain. FISCHER, BÖTTGER and

ECHTERNACHT (1941) nevertheless determined the molecular weight on a preparation from pancreas, which, however, had been produced by treatment with alkali and may have been damaged in that process. From diffusion experiments, they estimated the molecular weight to about 10 000, corresponding to about 30 mononucleotides.

From another material, namely virus, some important results have been obtained. COHEN and STANLEY (1942) produced from tobacco mosaic virus a polyribose nucleotide which, in a fresh condition, had a molecular weight of about 300 000, but which spontaneously broke down to 61 000. On treatment with alkali in cold the molecular weight fell to about 15 000, the axis ratio being estimated at 1/10. These authors presume that the polyribose nucleotide in the natural form has thread-like molecules. As, on polymerization, the molecular unions seem to permit a deviation from the linear form, a reduction in asymmetry apparently occurs.

B. Material.

a. *Polyribodesose nucleotides.*

The thymonucleic acid preparations were produced by HAMMARSTEN in accordance with the method indicated by him (HAMMARSTEN 1924). This original preparation (here termed TN I) as well as a preparation which had been further purified (here termed TN II) were used in this investigation.

TN I is the same preparation as that studied by HAMMARSTEN (1924). Its protein content is about 1 %. The preparation used was air-dried and had 15.31 % moisture. The phosphorus content was 7.74 %. A solution with 1.0 g/100 ml had a chloride concentration corresponding to 0.001 normality.

TN II was made in the same way as TN I, but was afterwards purified with a method elaborated by HAMMARSTEN (to be published). According to this method, the preparation is dissolved in hydrous phenol and is ultracentrifuged at 50 000

r. p. m. The preparation is then free from protein. The preparation used for this investigation was air-dried and contained 15.75 % moisture. The analyses showed 12.28 % nitrogen and 7.57 % phosphorus. A solution with 1.0 g/100 ml contained chlorides in a concentration of 0.0025 normality.

The partial specific volume was determined for TN II at a concentration of 0.7 per cent. by weight. The value 0.50 was found. As the densities were determined with a possible error in the 4th decimal, the accuracy was not very high, but sufficient for this investigation. The value was also considered applicable to the other preparation used, as well as to weaker concentrations.

b. Polyribose nucleotides.

The preparation was made from pancreas by HAMMARSTEN according to a method elaborated by him. The preparation is free from protein and high-polymeric with a buffer capacity, between pH 5 and pH 7, of 0.12—0.14 milliequivalents per mg-atom of phosphorus. The analyses showed 5.14 % nitrogen and 8.05 % phosphorus. The moisture content was 9.22 % in air-dried state. The preparation had been precipitated with hydrochloric acid and ethyl alcohol in cold and was dissolved with a gentle addition of alkali.

The purification method and analysis data for these nucleotides (as in the case of TN II) will be published by E. HAMMARSTEN.

c. Other preparations.

Some preliminary studies were made of other nucleotides, namely a sodium salt of uridylic acid and commercial nucleic acid from yeast (Merck).

The uridylic acid preparation was made by JORPES. It contained 6.87 % nitrogen and 6.84 % phosphorus.

In investigating the serviceableness of the method for nucleotides, Merck's nucleic acid preparation from yeast was first tested. It had, however, been produced by treatment with

alkali and contained nucleotides with very different degrees of polymerization. But, as it was used for many investigations and is thus of a certain general interest, the results have nevertheless been reported here.

At the laboratory, phosphorus is determined according to the method of PREGL, and nitrogen according to the micro method of KJELDAHL. The moisture is computed from the loss of weight on drying to a constant weight at 90° to 100° C., in a vacuum over phosphorus pentoxide.

The nucleotides examined were always used in the form of sodium salts and are referred to as such even where, for the sake of brevity, they are mentioned as free acids.

CHAPTER X

The dielectric constant for aqueous solutions of polynucleotides at different concentrations

Dielectric studies have not previously been made in regard to polynucleotides. It is of interest, however, to note that HAUSSER and KINDER (1938), in connection with a dielectric study of cozymase, made a number of determinations on yeast adenylic acid, etc. They adopted »Drude's second method» (1897), at a wave-length of about 2 metres. For yeast adenylic acid they found in a $1/69$ molar solution a molar increment of 85. From this it can be inferred that the measured increase of DC (when the adenylic acid had been dissolved in water and pH was 2.85) was about 1.24 DC units. By the addition of alkali, pH was varied. The increment diminished approximately linearly with increasing pH, being at pH 4.35 about 28, *i. e.* the rise of DC was about 0.24 DC units. The conductivity was high (about 10^{-3} ohm $^{-1}$ cm $^{-1}$) and was compensated with dilute solutions of hydrochloric acid.

BLÜH and KROCZEK (1934), who had likewise adopted Drude's second method, have shown that such a conductivity in the solution can apparently shift DC several units. To compensate this by corrections and, by comparison with dilute hydrochloric acid, to ensure rises of 0.24 to at most 1.24 DC units is by no means easy. Moreover, though the absolute value of the control solutions is unknown, it is nevertheless reckoned as the DC of the water. Even a relatively small shifting of DC owing to the action of the hydrochloric acid will considerably affect the result. For extended yeast adenylic acid, they estimate the distance between the charges at about

Table 5.

*DC of uridylic acid in aqueous solutions at different concentrations.
pH = 7.3. Temp. 20° C. Wave-length 1200 m.*

Concentration		DC	Incidental error of DC	Increment per mg-atom phosphorus per litre
g/100 ml	mg-atoms phosphorus per litre			
0.05	1.1	82.9	0.33	2.4
0.10	2.2	84.1	0.32	1.7
0.20	4.4	85.3	0.35	1.1
0.25	5.5	85.1	0.46	0.9

10 Å. From this it can be inferred that HAUSSER and KINDER made their determinations within the dispersion range of yeast adenylic acid, whence merely a minor part of a possible increment there could be observed.

In connection with the study of polynucleotides, it seemed to be of interest to make a comparative investigation of some mononucleotide. Some determinations were accordingly made on the sodium salt of uridylic acid at different concentrations. The solutions were prepared from a stock solution with pH 7.3 (the slight shifting of pH during dilution was not compensated).

As shown by Table 5, uridylic acid, at about pH 7.3, has a very high molar increment, which, however, within the range of concentration examined, is not constant. As shown in Fig. 15, the curve gradually bends off at higher concentrations.

In order to test the serviceableness of the method, a number of determinations were at first made on commercial yeast nucleic acid. It was examined at different pH between 5.8 and 6.65. Within this pH range, no difference in increment could be observed. As, despite the lack of homogeneity in the preparation, the determinations are of interest, some of them will be reproduced here.

As shown by Table 6, commercial yeast nucleic acid has a high increment, though somewhat lower than for uridylic acid.

Table 6.

*DC of nucleic acid from yeast (Merck) in aqueous solutions at different concentrations. pH = 5.s—6.65. Temp. 20° C.
Wave-length 1200 m.*

Concentration g/100 ml	DC	Incidental error of DC
0.125	83.8	0.13
0.25	85.9 ¹	—
0.33	87.7	0.46
0.5	88.5	0.64
1.0	89.7	0.21
2.0	92.0 ²	—

¹ mean of 8 values.

² mean of 6 values.

The increment is fairly constant at concentrations under 0.3 g/100 ml (see Fig. 15).

A. Polyribose nucleotides.

The preparation, as already mentioned, was precipitated as free acid. The weights are therefore given in grams of free acid

Table 7.

*DC of polyribose nucleotides in aqueous solutions at different concentrations. pH = 5.s. Temp. 20° C.
Wave-length 1200 m.*

Concentration g/100 ml	mg-atoms phosphorus per litre	DC	Incidental error of DC	Increment per mg atom phosphorus per litre
0.15	3.9	83.4	0.34	0.79
0.25	6.5	84.9	0.19	0.71
0.33	8.6	86.3	0.42	0.70
0.5	13.0	88.3	0.67	0.62

per 100 ml. The solution was prepared from a 0.5 % stock solution, where pH was adjusted, by the addition of alkali, to about 5.8. The measuring values are given in Table 7.

The DC for different concentrations is shown in Fig. 15, which, for purpose of comparision, also gives the concentration curves for uridylic acid and commercial yeast nucleic acid.

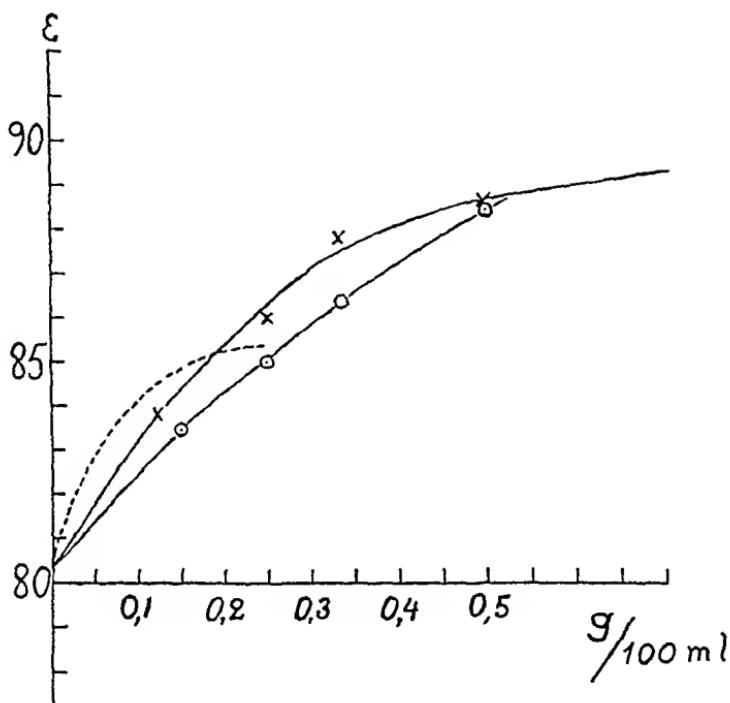


Fig. 15. DC of polyribose nucleotide in aqueous solutions at different concentrations.

— ○ — denotes polyribose nucleotide. By way of comparison, the broken line for uridylic acid and — × — for nucleic acid from yeast have been included.

Results: Polyribose nucleotides have an increment which is considerably lower than that of commercial yeast nucleic acid and especially uridylic acid. On the other hand, the increment is fairly constant within the range of concentration examined.

B. Polyribodesose nucleotides.

In view of the effect of time on the viscosity and conductivity of these nucleotides, shown by HAMMARSTEN in 1924 (see p. 77), the possibility that also the DC varied with time was investigated. The solutions were examined 5—48 hours after preparation, and during this period no dependence on time could be observed.

Results: The values determined in respect of the thymonucleic acid preparations examined will be found in Tables 8 and 9.

Table 8.

DC of polyribodesose nucleotide, TN I, in aqueous solutions at different concentrations. Temp. 20° C. Wave-length 1200 m.

Concentration		DC	Incidental error of DC	Increment per mg atom phosphorus per litre
g/100 ml	mg atoms phosphorus per litre			
0.025	0.62	89.5	0.31	15
0.05	1.2	95.0	0.11	12
0.075	1.9	100.0	0.38	11
0.1	2.5	101.2	0.11	8.4
0.125	3.1	99.5 ¹	—	6.2
0.19	4.7	93.5	0.18	2.8
0.25	6.2	91.1	0.38	1.7

¹ mean of 7 values.

TN I at very low concentrations already shows an extremely high increment, which even for concentrations under 0.1 g/100 ml is not quite constant. At a concentration of 0.1 g/100 ml a very marked change in the curve occurs, so that DC at higher concentrations rapidly falls (see Fig. 16).

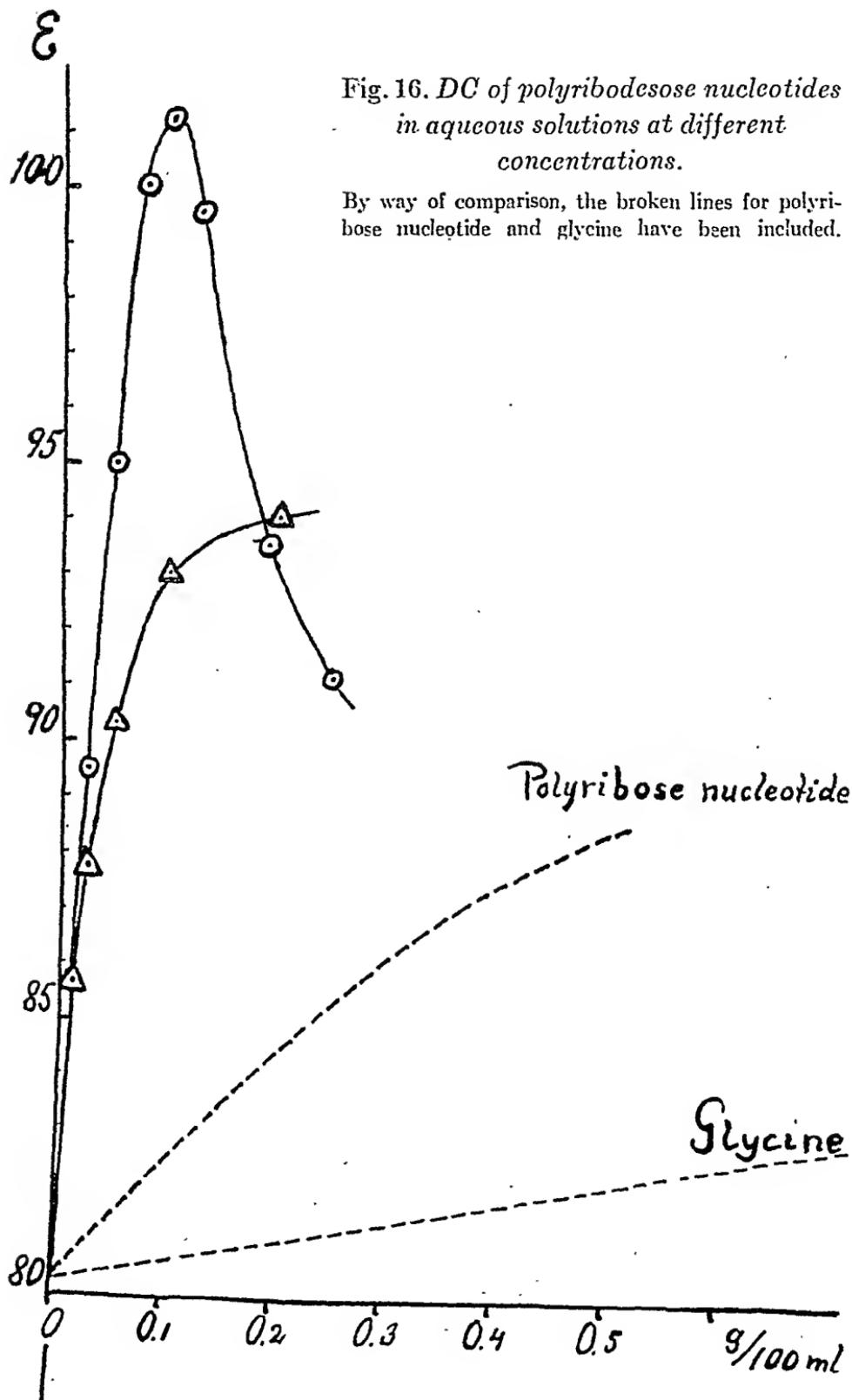


Table 9.

DC of polyribodesose nucleotide, TN II, in aqueous solutions at different concentrations. Temp. 20° C. Wave-length 1200 m.

Concentration		DC	Incidental error of DC	Increment per mg-atom phosphorus per litre
g/100 ml	mg-atoms phosphorus per litre			
0.0125	0.31	85.7	0.16	18
0.025	0.61	87.8	0.10	12
0.05	1.2	90.3	0.12	8.2
0.10	2.4	93.0	0.13	5.2
0.20	4.9	94.0	0.25	2.8

TN II also has a very high increment, which at some hundredths per cent. of the solution deviates from the linear course. At a concentration of 0.1 g/100 ml, the increment falls much more rapidly. On the concentration curve (Fig. 16) this is indicated by the rather sharp bend.

As mentioned before, neutral salts have a marked effect on the viscosity of polyribodesose nucleotides (p. 77). Also DC is considerably lowered by small quantities of potassium chloride. The amounts of neutral salts in the preparations are, however, so minute that they may be ignored.

C. Comments.

All kinds of nucleotides were found to belong to the relatively small group of substances which have a (positive) increment.

As regards the sodium salt of uridylic acid, the increase in DC is many times greater than was found by HAUSSER and KINDER for yeast adenylic acid. As we are concerned here with analogous substances, this disparity is remarkable, and it does not seem possible to get the results to tally except on the assumption of a critical wave-length of several metres.

Commercial yeast nucleic acid has certainly a greatly varying degree of polymerization and, also in dielectric respects,

holds an intermediate position between uridylic acid and polyribose nucleotides.

The increment for the sodium salt of polyribose nucleotides, which, to judge by the low buffer capacity, has a relatively high degree of polymerization, is lower than for uridylic acid and for Merck's yeast nucleic acid. This corresponds to a minor polarity in the molecule, due to the linkage of the mono-nucleotides on polymerization, so that the moments to some extent neutralize one another. The increment at different concentrations is relatively constant, which is also in conformity with the less marked dipole association.

Polyribodesose nucleotides, at low concentrations (under 0.1 g/100 ml), show an extreme rise of DC for water. The molar increment (reckoned according to a molecular weight of 200 000) will be more than ten million.

A noteworthy phenomenon is the very marked change that occurs at a concentration of 0.1 g/100 ml, and which is particularly pronounced as regards HAMMARSTEN's original preparation, where a fall of DC is actually observed. Such a marked association must manifest itself in the form of a change in viscosity. This effect was observed by HAMMARSTEN as far back as 1924, when he found that the viscosity began to increase more rapidly at a concentration approximately corresponding to 0.2 g/100 ml (see Fig. 14, p. 78). The different concentrations at which the changes occur do not conflict with one another, as it must be assumed that the determinations of DC are much more sensitive and react more rapidly to an association of non-polar type. The tendency to form units, which was observed also by PEDERSEN (1940) in ultracentrifuging, was the basis of the explanation given by LINDERSTRØM-LANG (1926) of HAMMARSTEN's volume effect. Were it not for the screen effect of the positive ions, the association, on the rise of the concentration, would soon result in precipitation.

CHAPTER XI

Anomalous dispersion of polyribodesose nucleotides in aqueous solution

In the very first investigations of polyribodesose nucleotides at different frequencies within the radio frequency range, it had already been ascertained that the units rotating in the alternating field had a considerably lower molecular weight than had been found, for example, by ultracentrifuging and determination of the double refraction of flow. By extending the range wave-length so that these minor units could be measured, facilities were afforded for increasing our knowledge of the polyribodesose nucleotides by determination of DC. On extension of the range to 2 Mc/s, an incipient dispersion could be shown for polyribodesose nucleotides, and with the fixed frequencies the course of the whole dispersion curve could be determined.

As regards the polyribose nucleotides, the wave-length range could not be extended so far, but down to 145 m no dispersion could be shown.

The determination of dispersion curves in electrically conductive solutions takes considerable time, whence the individual measuring points in practice are comparable with isolated and independent values. TN I as well as TN II were examined at a concentration of 0.1 g/100 ml.

The changes in DC at different frequencies for TN I and TN II are best shown in Fig. 17. As will be seen, DC falls at shorter wave-lengths and the shape of the curves indicates regular dispersions. Although at long wave-lengths the preparations show considerably different values, the difference is equalized, and at high frequencies the solutions have approxi-

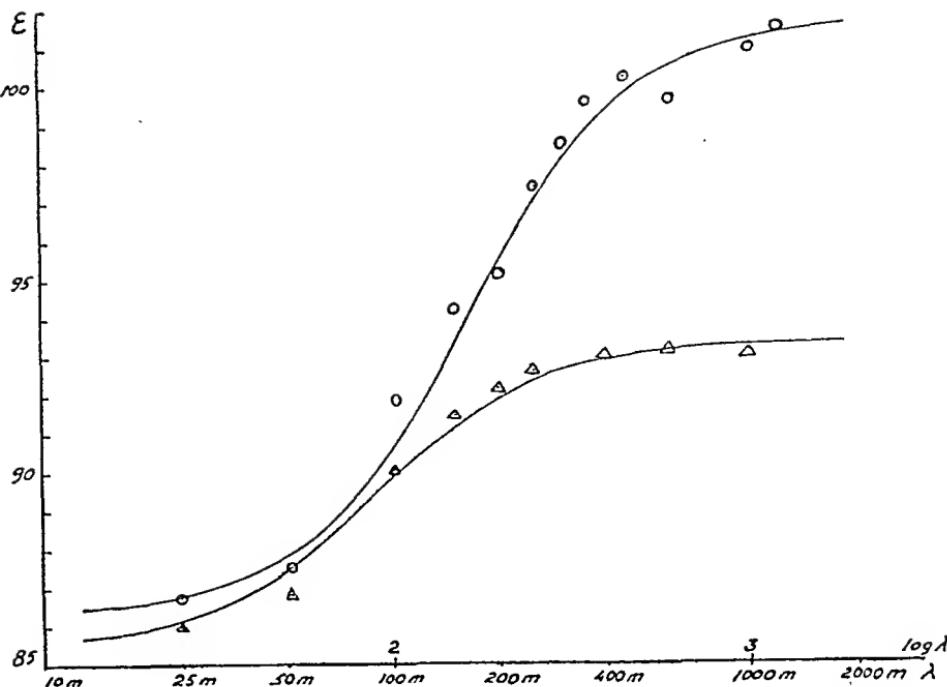


Fig. 17. Anomalous dispersion of polyribodesose nucleotides.

○ denotes TN I.

△ denotes TN II.

mately the same DC, which, however, is considerably higher than that of water. Thus polyribodesose nucleotides have at least one additional anomalous dispersion.

For the computation of the molecular weight — which is of special interest in this connection —, an expression for the critical wave-length λ_c was obtained from the equations (16) and (18) namely

$$\lambda_c = \lambda \sqrt{\frac{\varepsilon_l - \varepsilon_\omega}{\varepsilon_\omega - \varepsilon_h}}$$

where λ is the wave-length and λ_c the critical wave-length (transferred from the corresponding frequencies). From the conformation of the curves, probable values of ε_l and ε_h were first assumed and, as approximate values, the following figures seemed probable:

	λ_c	ε_l	ε_h
TN I . . .	$1.63 \cdot 10^4$ cm	101.3	86.4
TN II . . .	$0.90 \cdot 10^4$ cm	93.2	85.6

On the basis of the assumed values, tests were made until λ_c in the equation (16 and 18) was approximately constant. The results then obtained were

	λ_c	ε	ε_h
TN I . . .	$1.50 \cdot 10^4$ cm	101.1	86.3
TN II . . .	$0.91 \cdot 10^4$ cm	93.2	85.6

The values are given in Tables 10 and 11. Considerable errors arise in computing λ_c from DC determinations at increasing distances from the centres of the curves. In such determinations regard is then paid only to the values in imme-

Table 10.

*DC of polyribodesose nucleotide, TN I, at different wave-lengths.
Temp. 20° C.*

Wave-length m	DC	Incidental error of DC	$\lambda_c \cdot 10^4$ (cm)	Weighting of λ_c
25	86.7	0.22	(1.50)	0
51.7	87.5	0.10	(1.74)	0
103.4	91.8	0.06	1.35	1
150	94.1	0.16	1.42	1
200	95.0	0.12	1.68	1
250	97.2	0.19	1.50	1
300	98.3	0.13	1.45	$\frac{1}{2}$
350	99.4	0.15	1.26	$\frac{1}{2}$
450	100.0	0.11	1.28	$\frac{1}{2}$
600	99.4	0.15	2.16	$\frac{1}{2}$
1000	100.7	0.18	(1.62)	0
1200	101.1	0.11	—	0

For the computation of λ_c it was assumed that $\varepsilon_l = 101.1$
 $\varepsilon_h = 86.3$

Found value: $\lambda_c = 150$ m.

Table 11.

*DC of polyribodesose nucleotide, TN II, at different wave-lengths.
Temp. 20° C.*

Wave-length m	DC	Incidental error of DC	$\lambda_c \cdot 10^4$ (cm)	Weighting of λ_c
25	86.0	0.21	(1.06)	0
51.7	86.8	0.20	1.20	$\frac{1}{2}$
103.4	90.0	0.27	0.88	1
150	91.4	0.17	0.84	1
200	92.2	0.14	0.82	$\frac{1}{2}$
250	92.6	0.12	0.73	0
400	92.9	0.18	(0.81)	0
600	93.0	0.14	(0.99)	0
1000	92.9	0.14	—	0

For the computation of λ_c it was assumed that $\epsilon_l = 93.2$
 $\epsilon_h = 85.6$

Found value: $\lambda_c = 91$ m.

diate proximity to λ_c , in order to obtain a mean value. The estimation of the weighting of different values was made according to ARRHENIUS (1940). This computation corresponds to an equalization of the errors in a horizontal direction on the curves, which is the most satisfactory procedure for the determination of λ_c .

For the purpose of control, the method of least squares was adopted. This, however, does not involve any distinct advantage, and the estimation of the errors will be chiefly a matter of judgment. The error percentages must be rather high; thus the author has estimated that the errors for the critical wavelength may amount to 15—20 %, though they probably do not exceed 10 %.

As the result of discussions, the author has found the following values of λ_c to be most probable:

TN I λ_c 155 m
TN II λ_c 91 m

The size of the rotating units was computed according to equation (23), 0.59 being used as a value for the specific volume. The molecular weight, M , of the rotating units will then be

$$\text{TN I } M = 15\ 500 \quad \text{TN II } M = 9\ 800$$

For these low molecular weights the form factor shows considerable deviation from the limit value. From X-ray data (ASTBURY and BELL, 1938) the axis ratio was computed to $\frac{1}{10}$ for TN I and $\frac{1}{6}$ for TN II. Corrections give the following probable values (and estimated errors)

$$\begin{aligned}\text{TN I } 15\ 500 - 3 \% &= 15\ 000 \pm 1\ 500 \\ \text{TN II } 9\ 800 - 10 \% &= 9\ 000 \pm 1\ 000.\end{aligned}$$

The dipole moments of the units, computed according to Debye, on the basis of the formula (11 b), will be

$$\begin{aligned}\text{TN I circa } 65 \text{ Debye} \\ \text{TN II circa } 38 \text{ Debye}\end{aligned}$$

The molecular weights and the relative dipole moments, within normal errors of measurement, are thus in the ratio of 2:1. It is therefore plausible to suppose that a larger polar unit has been divided into two parts. How far impurities (protein) in the original preparation may have conducted to this result is uncertain. Dielectric studies can merely reveal the difference, without giving any definite information as to how the change has been brought about. In order to settle this matter, also other methods are required.

Summary, Part III

The dielectric study of polynucleotides has shown that, in the form of sodium salts in aqueous solution, they have very high increments. The thymonucleic acid preparation, the production of which was indicated by HAMMARSTEN in 1924, has shown the highest increment, but also the protein-free preparation subsequently produced by him shows an increment of very high magnitude. Owing to a very marked tendency to association, however, the increments are tolerably constant only in extremely dilute solutions. No exact figures for the increment should therefore be given. The order of magnitude should be sufficient to show the markedly polar character of the molecules.

Polyribose nucleotides likewise have a large increment, though considerably lower than that of the polyribodesose nucleotides. This is presumably due to the fact that, in the building-up of the polyribose nucleotides, the mononucleotides are linked together so that their dipole moments partly neutralize one another.

This seems to be supported by some determinations of uridylic acid, which in very weak concentrations has a higher increment, reckoned per phosphorus atom. The increment, however, does not agree with that found by HAUSSER and KINDER for adenylic acid, and it does not seem possible to get the results to tally, except on the assumption of a critical wave-length of several metres.

A heterogeneous preparation with a greatly varying degree of polymerization has been found to hold an intermediate position between uridylic acid and polyribose nucleotides.

In conjunction with the polar character of the polynucleotides, a very marked tendency to association is manifested, which as regards polyribodesose nucleotides is so pronounced that for one of the preparations (TN I) it showed a fall of the DC at concentrations over 0.1 g/100 ml.

As regards polyribodesose nucleotides, a dispersion within the short-wave range was shown. It was found that the critical wave-lengths were not the same for the two preparations examined. The less pure preparation, TN I, had a dispersion corresponding to a molecular weight of about 15 000, whilst the protein-free preparation, TN II, was estimated to have a molecular weight of about 9 000.

It is of special interest that the units rotating in the alternating field have molecular weights which, within the same grade of concentration as those examined with the ultracentrifuge (PEDERSEN, 1940), were then found to have a molecular weight of about 200 000. Which of the two figures corresponds to the real molecular weight of thymonucleic acid, cannot be stated with certainty, but that found by the dielectric investigation is a minimum weight. In view of the marked tendency to association shown in many ways by thymonucleic acid, it seems quite possible that higher molecular weights may be recorded in associated complexes. In that case an end-to-end association must be assumed for correspondence with the results of the double refraction of flow (SIGNER, CASPERSSON and HAMMARSTEN, 1938). It cannot, however, be determined whether this is merely a case of association, or whether the thymonucleic acid molecule is built up of a chain of freely rotating units.

The dispersion curves permitted a computation of relative dipole moments according to the method of Debye. For TN I, 65 Debye units are obtained, for TN II about 38. As regards the causes of the difference between the preparations, it is as yet too early to express an opinion. The effect even of a very minute amount of protein may be considerable, and cannot be judged before nucleproteins have been investigated.

Polyribose nucleotides showed no dispersion at wave-lengths down to 145 m.

The difference between polyribose and polyribodesose nucleotides in regard to polarity and size of the rotating units seems to be of interest for the study of their biological functions, with which we are now fairly well acquainted (HAMMARSTEN 1924, 1939, CASPERSSON 1936, 1940, 1941).

General Summary and Conclusions

With a view to the determination of dielectric constants in polynucleotides at variable frequencies within the radio frequency range, the author elaborated an apparatus which is based, in principle, on the ellipsoid method indicated by Fürth.

It was found that force methods were the only ones that could be seriously contemplated for the purpose and, in choosing between the ellipsoid and the electrometer method, it was considered that the former for several reasons was decidedly preferable.

The apparatus was thoroughly tested and controlled and was found well adapted for the study of polynucleotides.

Investigation with this method has shown that polynucleotides in aqueous solution have very high (positive) increments. They have thus been found to belong to the relatively very small group of substances with this property, within which biologically important substances constitute the majority. The polyribose nucleotides were found to show considerably less polarity than the polyribodesose nucleotides, which have molar increments of a previously unknown order of magnitude.

As regards polyribodesose nucleotides, a dispersion was shown and its course determined. On the basis of the determinations made, the molecular weights were computed. In this regard, a dielectric study yields information regarding polynucleotides which cannot be obtained with any other method. The estimated molecular weights are in fact considerably lower than those found with other methods. This must be interpreted to signify that the molecules or molecular aggregates are actually built up of smaller units, which can be discovered in this way.

A purified and protein-free polyribodesose nucleotide preparation yielded a markedly lower molecular weight and rela-

tive dipole moment than those which had not been so highly purified.

In contradistinction from polyribodesose nucleotides, no dispersion was shown by polyribose nucleotides down to 145 m wave-length.

Generally speaking, the dielectric method has thus proved to be very valuable in studying substances such as the poly-nucleotides.

This investigation has been financially supported by grants from »Stiftelsen Thérèse och Johan Anderssons Minne» and from ASTRA (Pharmaceutical Chemists' Manufacturing Company).

References

ALLEN, F. W., Ann. Rev. Biochem. 1941, *X*, 221.

ARRHENIUS, S., Nova Acta Reg. Soc. Sci. Upsal. IV, 1940, *12*, No. 5.

— »The Svedberg 1884—1944», 1944, 419.

ASTBURY, W. T., and F. O. BELL, Cold Spring Harbour Symp. Q. Biol. 1938, *6*, 109.

— — Nature 1938, *141*, 747.

BJÖRNSTÅHL, Y., Z. Physik 1941, *118*, 257.

— and K. O. SNELLMAN, Kolloid-Z. 1937, *78*, 271.

BLÜH, O., Z. Physik 1926, *27*, 226.

— Z. physik. Chem. 1934, B *27*, 263.

— and J. KROCZEK, Z. physik. Chem. 1934 B, *27*, 270.

BREDERECK, H., Fortschritte d. Chem. org. Naturstoffe 1938, *121*.

CARMAN, A. P., Phys. Rev. 1924, *24*, 396.

— and C. C. SCHMIDT, Phys. Rev. 1927, *30*, 922.

— — Phys. Rev. 1928, *31*, 157.

— and K. O. SMITH, Phys. Rev. 1929, *34*, 1042.

— O. B. YOUNG and K. O. SMITH, Phys. Rev. 1929, *34*, 1940.

CASPERSSON, T., Skandinav. Arch. Physiol. Suppl. 1936, *8*, 73.

— Naturwissenschaften 1941, *29*, 33.

— Chromosoma 1939/40, *1*, 147.

COHEN, S. S., and W. M. STANLEY, J. Biol. Chem. 1942, *144*, 589.

COHN, E., Physik. Z. 1931, *32*, 687.

COLE, K. S., and H. J. CURTIS, Rev. Sci. Instr. 1937, *8*, 333.

DEBYE, P., »Polare Molekeln», Leipzig 1929.

DRAKE, F. H., G. W. PIERCE and M. T. DOW, Phys. Rev. 1930, *35*, 613.

DRUDE, P., Wied. Ann. 1893, *48*, 593.

— Z. phys. Chem. 1897, *23*, 267.

DUNNING, W. J., and W. J. SHUTT, Trans. Far. Soc. 1938, *34*, 479.

FALKENHAGEN, H., »Electrolytes» 1934. Clarendon Press (Oxford).

FISCHER, G., and W.-D. SCHAFFELD, Ann. d. Physik 1936, [5], *25*, 450.

FISCHER, F. G., I. Böttger and H. Echternacht, Z. physiol. Ch. 1941, *271*, 246.

FÜRTH, R., Z. Physik 1924, *22*, 98.

GRAFFUNDER, W., and R. WEBER, Ann. d. Physik 1931, [5], *9*, 887.

GRUBB, H. M., and H. HUNT, J. Am. Chem. Soc. 1939, 61, 565.
GULLAND, J. M., J. Chem. Soc. 1938, 1722.
HAMMARSTEN, E., Biochem. Z. 1924, 144, 383.
— J. Mount Sinai Hospital 1939, VI: 3, 115.
HARRINGTON, J., Phys. Rev. 1916, 8, 581.
HAUSSER, I., and E. KINDER, Z. physik. Chem. 1938, B. 41, 142.
HEDESTRAND, G., Z. physik. Chem. 1928, A. 135, 36.
— Z. physik. Chem. 1929, B. 2, 428.
HOLLÄNDER, A., Lotos 1930, 78, 9.
JONES, G., and S. M. CHRISTIAN, J. Am. Chem. Soc. 1935, 57,
272.
KOCKEL, L., Ann. d. Physik. 1925, [4], 77, 417.
LIN, T., Ann. d. Physik 1936, [5], 26, 495.
LINDERSTRØM-LANG, K., Compt.rend.trav.lab. Carlsberg, 16: 6.
MILICKA, O., and A. SLAMA, Ann. d. Physik 1931, [5], 8, 663.
NERNST, W., Wied. Ann. 1896, 57, 209.
ONCLEY, J. L., J. Am. Chem. Soc. 1938, 60, 1115.
ONSAGER, L., J. Am. Chem. Soc. 1936, 58, 1486.
ORTHMANN, W., Ann. d. Physik 1931, [5], 9, 537.
PECHHOLD, R., Ann. d. Physik 1927, [4], 83, 427.
PEDERSEN, K. O. in SVEDBERG and PEDERSEN »The Ultracentrifuge» 1940, 443. Clarendon Press (Oxford).
PERRIN, F., (cited from Arrhenius, 1940), Journ. de Phys. et le
Radium 1934, [7], 5, 497.
RAJEWSKY, B., Ergebnisse der biophysikalischen Forschung 1938,
I, (Ultrakurzwellen).
SIGNER, R., T. CASPERSSON and E. HAMMARSTEN, Nature 1938,
141, 122.
SHUTT, W. J., Trans. Far. Soc. 1934, 30, 893.
WALDEN, P., and O. WERNER, Z. physik. Chem. 1927, 129, 405.
— H. ULLICH and O. WERNER, Z. physik. Chem. 1925, 115, 177.
WHETHAM, W. C. D., Phil. Trans. 1900, 194, 321.
WILLIAMS, J. W., and M. A. ELLIOT, J. Am. Chem. Soc. 1939,
61, 718.
WYMAN, J. JR., J. Am. Chem. Soc. 1933, 55, 4116.
— J. Am. Chem. Soc. 1936, 58, 1482.
— and T. L. MC MEEKIN, J. Am. Chem. Soc. 1933, 55, 908, 915.
ZAHN, H., Phys. Z. 1927, 28, 916.

